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**HUMAN HEPATITIS B VIRUS CORE PROTEINS AS VACCINE PLATFORMS  
AND METHODS OF USE THEREOF**

The invention was made in part with Government support by the National Institutes of  
5 Health, Grants RO1 AI020720 and RO1 AI049730. As such, the Government has certain rights  
in the invention.

**FIELD OF THE INVENTION**

The present invention relates to hepatitis virus core proteins and nucleic acids. In  
10 particular, the present invention provides compositions and methods comprising recombinant  
hepatitis virus core proteins or nucleic acids for use in vaccine formulations.

**BACKGROUND OF THE INVENTION**

The ability to map neutralizing B cell epitopes on protein and carbohydrate antigens has  
15 created much interest in the potential use of these hapten-like antigens in vaccine development.  
There are a number of advantages to the use of peptides and small well-defined oligosaccharides  
(OS) for subunit vaccine design, including for example, chemical purity and safety, ease of  
production, cost, stability, defined and targeted B and/or T cell epitopes and mutability. The  
promise of the hapten-like technology hasn't been fully realized because efficient and  
20 reproducible methods for the delivery of these small epitopes to the immune system are lacking.  
Peptidic and OS antigens often require conjugation to an immunogenic carrier in order to provide  
efficient T cell help for antibody producing B cells, as peptide antigens often do not contain  
helper T (Th) cell epitopes and carbohydrate antigens are not recognized by T cells.

The particulate human hepatitis B virus (HBV) core protein (HBcAg) has been utilized as  
25 a carrier platform as it possesses many of the characteristics uniquely required for the delivery of  
weak immunogens to the immune system (See, Pimpers and Grens, *Intervirology*, 44:98-114,  
2001). Although the HBcAg is highly immunogenic, the existing HBcAg-based platform  
technology has a number of serious theoretical and practical limitations. For example, less than  
50% of foreign epitopes can be accommodated by the HBcAg platform because of adverse  
30 effects on particle assembly (Jegerlehner *et al.*, *Vaccine*, 20:3104, 2002 and PCT/US01/25625);  
use of the HBcAg compromises the use of the anti-HBc diagnostic assay; and immune tolerance

to HBcAg in individuals chronically infected with HBV (300-400 million worldwide) limits immunogenicity in this population. Thus, there is a profound need in the art for particulate carrier platforms capable of delivering a wide variety of heterologous peptide and oligosaccharide epitopes in an immunogenic form. This need is particularly acute in the event  
5 the vaccine recipient is chronically infected with or suspected to be infected with HBV.

## SUMMARY OF THE INVENTION

The present invention relates to hepatitis virus core proteins and nucleic acids. In particular, the present invention provides compositions and methods comprising recombinant  
10 hepatitis virus core proteins or nucleic acids for use in vaccine formulations.

In particular, the present invention provides compositions comprising a heterologous antigen linked to the amino acid sequence set forth in SEQ ID NO:38, the amino acid sequence comprising a loop region. In some embodiments, the heterologous antigen is inserted at a position within the loop region. In preferred embodiments, the position within the loop region is  
15 chosen from amino acid residues 77, 78, 81, and 82. In another embodiment, the position within the loop region is at amino acid residue 76. In further embodiments, the heterologous antigen is inserted at a position outside of the loop region. In preferred embodiments, the position outside the loop region is chosen from amino acid residues 71, 72, 73, 74, 75, 83, 84, 85, 92, N-terminal and C-terminal. In another embodiment, the position outside the loop region is at amino acid  
20 residue 44. In still further embodiments, the heterologous antigen is inserted at a position within the loop region, and in a position outside the loop region. The present invention also provides composition in which the heterologous antigen is conjugated to the amino acid sequence. In preferred embodiments, the heterologous antigen comprises at least one B cell epitope. In further preferred embodiments, the heterologous antigen comprises at least one T helper cell  
25 epitope. In exemplary embodiments, the heterologous antigen is chosen from but not limited to human immunodeficiency virus antigen, feline immunodeficiency virus antigen, *Plasmodium* parasite antigen, influenza virus antigen, *Staphylococcus* bacterium antigen, cholesteryl ester transfer protein antigen, major histocompatibility complex antigen, cytokine antigen, amyloid  $\beta$ -peptide antigen, peanut allergen antigen, latex allergen hevein antigen, brown shrimp allergen  
30 antigen and major grass pollen allergen antigen.

The present invention also provides compositions comprising a heterologous antigen linked to the amino acid sequence set forth in SEQ ID NO:38, the amino acid sequence comprising a loop region and further comprising from 1 to 100 amino acids at the carboxy end of residue I<sup>149</sup>. In some preferred embodiments, the 1 to 100 amino acids is chosen from R<sup>150</sup>, C<sup>150</sup>, K<sup>150</sup>, A<sup>150</sup>, R<sup>150</sup>R<sup>151</sup>C<sup>152</sup>, and SEQ ID NOS:2-20. In other preferred embodiments, the 1 to 100 amino acids is chosen from SEQ ID NOS:22-36. In still further preferred embodiments, the 1 to 100 amino acids is chosen from SEQ ID NOS:42-56. Additionally, in particularly preferred embodiments, the heterologous antigen linked to the amino acid sequence set forth in SEQ ID NO:38, comprises a particle having a diameter of 25 to 35 nm. In some embodiments, the amino acid sequence further comprises at least one immune enhancer sequence. The immune enhancer sequence is operably linked to the C-terminus of the amino acid sequence in some embodiments.

In exemplary embodiments, the immune enhancer sequence is chosen from but not limited to unmethylated CpG dinucleotides, CD40 ligand, complement C3d fragment, B cell activating factor, and soluble gene product of lymphocyte activation gene-3. Also provided are

embodiments further comprising woodchuck hepatitis virus core antigen chosen from wild type woodchuck hepatitis virus core antigen and modified woodchuck hepatitis virus core antigen lacking a heterologous antigen. In some embodiments, the composition further comprises one or more compounds chosen from adjuvant, diluent and carrier. In related embodiments, the adjuvant is chosen from but not limited to aluminum hydroxide, aluminum phosphate, squalene and mineral oil. Moreover, the present invention provides a nucleic acid sequence encoding a heterologous antigen linked to the amino acid sequence set forth in SEQ ID NO:38. Related embodiments provide an expression vector comprising a nucleic acid sequence encoding a heterologous antigen linked to the amino acid sequence set forth in SEQ ID NO:38.

Additionally, the present invention provides compositions comprising a heterologous antigen linked to an amino acid sequence which is at least 95% identical to the sequence set forth in SEQ ID NO:38, the amino acid sequence comprising a loop region and forming a particle having a diameter of 25 to 35 nm. In some embodiments, the heterologous antigen comprises at least one modification chosen from insertion of at least one acidic amino acid residue, and substitution of at least one acidic amino acid residue. In some preferred embodiments, the at least one acidic amino acid residue comprises from 1 to 10 residues of one or both of aspartic acid and glutamic acid. In other preferred embodiments, the amino acid sequence comprises at

least one modification chosen from insertion of at least one acidic amino acid residue, and substitution of at least one acidic amino acid residue. In some preferred embodiments, the at least one acidic amino acid residue comprises from 1 to 10 residues of one or both of aspartic acid and glutamic acid.

5       Also provided by the present invention are compositions comprising the amino acid sequence set forth in SEQ ID NO:38, the amino acid sequence comprising a loop region. In some embodiments, the amino acid sequence further comprises from 1 to 100 amino acids (excluding the wild type C-terminus set forth in SEQ ID NO:2) at the carboxy end of residue I<sup>149</sup>. In some preferred embodiments, the 1 to 100 amino acids is chosen from R<sup>150</sup>, C<sup>150</sup>, K<sup>150</sup>, A<sup>150</sup>,  
10      R<sup>150</sup>R<sup>151</sup>C<sup>152</sup>, and SEQ ID NOS:3-20. In other preferred embodiments, the 1 to 100 amino acids is chosen from SEQ ID NOS:22-36. In still further preferred embodiments, the 1 to 100 amino acids is chosen from SEQ ID NOS:42-56. Additionally, in some embodiments, the amino acid sequence further comprises at least one immune enhancer sequence. In preferred embodiments, the immune enhancer sequence is operably linked to the C-terminus of the amino acid sequence.  
15      In exemplary embodiments, the immune enhancer sequence is chosen from but not limited to unmethylated CpG dinucleotides, CD40 ligand, complement C3d fragment, B cell activating factor, and soluble gene product of lymphocyte activation gene-3. Also provided is a nucleic acid sequence encoding the amino acid sequence set forth in SEQ ID NO:38, the amino acid sequence comprising a loop region. Related embodiments provide an expression vector  
20      comprising the nucleic acid sequence encoding the amino acid sequence set forth in SEQ ID NO:38. Additionally, compositions further comprising a modified woodchuck hepatitis virus core antigen comprising a heterologous antigen are provided. In some particularly preferred embodiments, compositions are provided comprising an amino acid sequence which is at least 95% identical to SEQ ID NO:38, the amino acid sequence comprising a loop region and forming  
25      a particle having a diameter of 25 to 35 nm.

Importantly, the present invention provides methods, comprising: providing: a mammalian subject; and a composition comprising one or more of a polypeptide comprising a heterologous antigen linked to the amino acid sequence set forth in SEQ ID NO:38, the amino acid sequence comprising a loop region, and an expression vector encoding the polypeptide; and  
30      administering the composition to the subject under conditions such that an immune response is generated. In some embodiments, the immune response comprises one or more of lymphocyte

proliferative response, cytokine response and antibody response. In some preferred embodiments, the cytokine response comprises IL-2 production. In further embodiments, the antibody response comprises at least three fold higher levels of antibody than that observed before administration of the at least one composition. In particularly preferred embodiments, the antibody response comprises production of IgG antibodies. In related embodiments, the IgG antibodies comprise an autoantibody. In some preferred embodiments, the composition further comprises one or more compounds chosen from adjuvant, diluent and carrier. In related embodiments, the adjuvant is chosen from but not limited to aluminum hydroxide, aluminum phosphate, squalene and mineral oil.

Also provided by the present invention are methods for producing an immunogenic composition, comprising: providing: a heterologous antigen; and a hepatitis virus core antigen; altering at least one of the heterologous antigen and the hepatitis virus core antigen, with a modification chosen from insertion of at least one acidic amino acid residue and substitution of at least one amino acid residue; and inserting the heterologous antigen of step b within the hepatitis virus core antigen of step b to produce a modified hepatitis virus core antigen; expressing the modified hepatitis virus core antigen under conditions suitable for producing particles having a diameter of 25 to 35 nm. In some embodiments, in the absence of the altering, expression of the modified hepatitis virus core antigen yields aggregates rather than particles. In other embodiments, in the absence of the altering, expression of the modified hepatitis virus core antigen yields 25 fold less particles than does expression of a wild type hepatitis virus core antigen. In some preferred embodiments, the at least one acidic amino acid residue comprises from 1 to 10 aspartic acid residues. In other preferred embodiments, the at least one acidic amino acid residue comprises from 1 to 10 glutamic acid residues. In related embodiments, the at least one acidic amino acid residue comprises at least one aspartic acid residue and at least one glutamic acid residue. In some embodiments, the insertion is in at least one position chosen from the N-terminus and the C-terminus of the heterologous antigen. In other embodiments, the substitution comprises a replacement of at least one non-acidic amino acid residue within the heterologous antigen, with the at least one acidic amino acid residue. In preferred embodiments, the altering produces a modified heterologous antigen with an isoelectric point in the range of 2.0 to 7.0. In a subset of these embodiments, the altering produces a modified heterologous antigen with an isoelectric point more preferably in the range of 3.0 to 6.0, and most preferably in the

range of 4.0 to 5.0. In a preferred embodiment, the hepatitis virus core antigen is a woodchuck hepatitis virus core antigen.

Additionally, the present invention provides compositions comprising a heterologous antigen linked to the amino acid sequence set forth in SEQ ID NO:40, the amino acid sequence comprising a loop region. In some embodiments, the heterologous antigen is inserted at a position within the loop region. In preferred embodiments, the position within the loop region is chosen from amino acid residues 77, 80, and 81. In another embodiment, the position within the loop region is at amino acid residue 76. In further embodiments, the heterologous antigen is inserted at a position outside of the loop region. In preferred embodiments, the position outside the loop region is chosen from amino acid residues 71, 72, 73, 74, 75, 82, 83, 84, 91, N-terminal and C-terminal. In another embodiment, the position outside the loop region is at amino acid residue 44. In still further embodiments, the heterologous antigen is inserted at a position within the loop region, and in a position outside the loop region. The present invention also provides composition in which the heterologous antigen is conjugated to the amino acid sequence. In preferred embodiments, the heterologous antigen comprises at least one B cell epitope. In further preferred embodiments, the heterologous antigen comprises at least one T helper cell epitope. In exemplary embodiments, the heterologous antigen is chosen from but not limited to human immunodeficiency virus antigen, feline immunodeficiency virus antigen, *Plasmodium* parasite antigen, influenza virus antigen, *Staphylococcus* bacterium antigen, cholesteryl ester transfer protein antigen, major histocompatibility complex antigen, cytokine antigen, amyloid  $\beta$ -peptide antigen, peanut allergen antigen, latex allergen hevein antigen, brown shrimp allergen antigen and major grass pollen allergen antigen.

The present invention also provides compositions comprising a heterologous antigen linked to the amino acid sequence set forth in SEQ ID NO:40, the amino acid sequence comprising a loop region and further comprising from 1 to 100 amino acids at the carboxy end of residue I<sup>148</sup>. In some preferred embodiments, the 1 to 100 amino acids is chosen from R<sup>149</sup>, C<sup>149</sup>, K<sup>149</sup>, A<sup>149</sup>, R<sup>149</sup>R<sup>150</sup>C<sup>151</sup>, and SEQ ID NOS:3-6, 22-36. In other preferred embodiments, the 1 to 100 amino acids is chosen from SEQ ID NOS:2, 7-20. In still further preferred embodiments, the 1 to 100 amino acids is chosen from SEQ ID NOS:42-56. Additionally, in particularly preferred embodiments, the heterologous antigen linked to the amino acid sequence set forth in SEQ ID NO:40, comprises a particle having a diameter of 25 to 35 nm. In some embodiments,

the amino acid sequence further comprises at least one immune enhancer sequence. The immune enhancer sequence is operably linked to the C-terminus of the amino acid sequence in some embodiments. In exemplary embodiments, the immune enhancer sequence is chosen from but not limited to unmethylated CpG dinucleotides, CD40 ligand, complement C3d fragment, B cell activating factor, and soluble gene product of lymphocyte activation gene-3. Also provided are embodiments further comprising ground squirrel hepatitis virus core antigen chosen from wild type ground squirrel hepatitis virus core antigen and modified ground squirrel hepatitis virus core antigen lacking a heterologous antigen. In some embodiments, the composition further comprises one or more compounds chosen from adjuvant, diluent and carrier. In related 10 embodiments, the adjuvant is chosen from but not limited to aluminum hydroxide, aluminum phosphate, squalene and mineral oil. Moreover, the present invention provides a nucleic acid sequence encoding a heterologous antigen linked to the amino acid sequence set forth in SEQ ID NO:40. Related embodiments provide an expression vector comprising a nucleic acid sequence encoding a heterologous antigen linked to the amino acid sequence set forth in SEQ ID NO:40.

15 Additionally, the present invention provides compositions comprising a heterologous antigen linked to an amino acid sequence which is at least 95% identical to the sequence set forth in SEQ ID NO:40, the amino acid sequence comprising a loop region and forming a particle having a diameter of 25 to 35 nm. In some embodiments, the heterologous antigen comprises at least one modification chosen from insertion of at least one acidic amino acid residue, and 20 substitution of at least one acidic amino acid residue. In some preferred embodiments, the at least one acidic amino acid residue comprises from 1 to 10 residues of one or both of aspartic acid and glutamic acid. In other preferred embodiments, the amino acid sequence comprises at least one modification chosen from insertion of at least one acidic amino acid residue, and substitution of at least one acidic amino acid residue. In some preferred embodiments, the at 25 least one acidic amino acid residue comprises from 1 to 10 residues of one or both of aspartic acid and glutamic acid.

Also provided by the present invention are compositions comprising the amino acid sequence set forth in SEQ ID NO:40, the amino acid sequence comprising a loop region. In some embodiments, the amino acid sequence further comprises from 1 to 100 amino acids 30 (excluding the wild type C-terminus set forth in SEQ ID NO:22) at the carboxy end of residue I<sup>148</sup>. In some preferred embodiments, the 1 to 100 amino acids is chosen from R<sup>149</sup>, C<sup>149</sup>, K<sup>149</sup>,

A<sup>149</sup>, R<sup>149</sup>R<sup>150</sup>C<sup>151</sup>, and SEQ ID NOS:3-6, 23-36. In other preferred embodiments, the 1 to 100 amino acids is chosen from SEQ ID NOS:2-20. In still further preferred embodiments, the 1 to 100 amino acids is chosen from SEQ ID NOS:42-56. Additionally, in some embodiments, the amino acid sequence further comprises at least one immune enhancer sequence. In preferred 5 embodiments, the immune enhancer sequence is operably linked to the C-terminus of the amino acid sequence. In exemplary embodiments, the immune enhancer sequence is chosen from unmethylated CpG dinucleotides, CD40 ligand, complement C3d fragment, B cell activating factor, and soluble gene product of lymphocyte activation gene-3. Also provided is a nucleic acid sequence encoding the amino acid sequence set forth in SEQ ID NO:40, the amino acid 10 sequence comprising a loop region. Related embodiments provide an expression vector comprising the nucleic acid sequence encoding the amino acid sequence set forth in SEQ ID NO:40. Additionally, compositions further comprising a modified ground squirrel hepatitis virus core antigen comprising a heterologous antigen are provided. In some particularly preferred embodiments, compositions are provided comprising an amino acid sequence which is at least 15 95% identical to SEQ ID NO:40, the amino acid sequence comprising a loop region and forming a particle having a diameter of 25 to 35 nm.

Importantly, the present invention provides methods, comprising: providing: a mammalian subject; and a composition comprising one or more of a polypeptide comprising a heterologous antigen linked to the amino acid sequence set forth in SEQ ID NO:40, the amino 20 acid sequence comprising a loop region, and an expression vector encoding the polypeptide; and administering the composition to the subject under conditions such that an immune response is generated. In some embodiments, the immune response comprises one or more of lymphocyte proliferative response, cytokine response and antibody response. In some preferred embodiments, the cytokine response comprises IL-2 production. In further embodiments, the 25 antibody response comprises at least three fold higher levels of antibody than that observed before administration of the at least one composition. In particularly preferred embodiments, the antibody response comprises production of IgG antibodies. In related embodiments, the IgG antibodies comprise an autoantibody. In some preferred embodiments, the composition further comprises one or more compounds chosen from adjuvant, diluent and carrier. In related 30 embodiments, the adjuvant is chosen from but not limited to aluminum hydroxide, aluminum phosphate, squalene and mineral oil.

Also provided by the present invention are methods for producing an immunogenic composition, comprising: providing: a heterologous antigen; and a hepatitis virus core antigen; altering at least one of the heterologous antigen and the hepatitis virus core antigen, with a modification chosen from insertion of at least one acidic amino acid residue and substitution of at least one acidic amino acid residue; and inserting the heterologous antigen of step b within the hepatitis virus core antigen of step b to produce a modified hepatitis virus core antigen; expressing the modified hepatitis virus core antigen under conditions suitable for producing particles having a diameter of 25 to 35 nm. In some embodiments, in the absence of the altering, expression of the modified hepatitis virus core antigen yields aggregates rather than particles. In other embodiments, in the absence of the altering, expression of the modified hepatitis virus core antigen yields 25 fold less particles than does expression of a wild type hepatitis virus core antigen. In some preferred embodiments, the at least one acidic amino acid residue comprises from 1 to 10 aspartic acid residues. In other preferred embodiments, the at least one acidic amino acid residue comprises from 1 to 10 glutamic acid residues. In related embodiments, the at least one acidic amino acid residue comprises at least one aspartic acid residue and at least one glutamic acid residue. In some embodiments, the insertion is in at least one position chosen from the N-terminus and the C-terminus of the heterologous antigen. In other embodiments, the substitution comprises a replacement of at least one non-acidic amino acid residue within the heterologous antigen, with the at least one acidic amino acid residue. In preferred embodiments, the altering produces a modified heterologous antigen with an isoelectric point in the range of 2.0 to 7.0. In a subset of these embodiments, the altering produces a modified heterologous antigen with an isoelectric point more preferably in the range of 3.0 to 6.0, and most preferably in the range of 4.0 to 5.0. In a preferred embodiment, the hepatitis virus core antigen is a ground squirrel hepatitis virus core antigen.

Additionally, the present invention provides compositions comprising a heterologous antigen linked to the amino acid sequence set forth in SEQ ID NO:58, the amino acid sequence comprising a loop region and further comprising from 1 to 100 amino acids at the carboxy end of residue I<sup>149</sup>, and wherein the 1 to 100 amino acids does not comprise C<sup>150</sup> or the sequence set forth in SEQ ID NO:42 (excluding C<sup>150</sup>, and the wild type C-terminus). In some preferred embodiments, the 1 to 100 amino acids is chosen from R<sup>150</sup>, K<sup>150</sup>, A<sup>150</sup>, R<sup>150</sup>R<sup>151</sup>C<sup>152</sup>, and SEQ ID NOS:3-6, 43-56. In other preferred embodiments, the 1 to 100 amino acids is chosen from

SEQ ID NOS:2, 7-20. In still further preferred embodiments, the 1 to 100 amino acids is chosen from SEQ ID NOS:22-36. Additionally, in particularly preferred embodiments, the heterologous antigen linked to the amino acid sequence set forth in SEQ ID NO:58, comprises a particle having a diameter of 25 to 35 nm.

5       In some embodiments, the heterologous antigen is inserted at a position within the loop region. In preferred embodiments, the position within the loop region is chosen from amino acid residues 77, 78, 81, and 82. In another embodiment, the position within the loop region is at amino acid residue 76. In further embodiments, the heterologous antigen is inserted at a position outside of the loop region. In preferred embodiments, the position outside the loop region is

10      chosen from amino acid residues 71, 72, 73, 74, 75, 83, 84, 85, 92, N-terminal and C-terminal. In another embodiment, the position outside the loop region is at amino acid residue 44. In still further embodiments, the heterologous antigen is inserted at a position within the loop region, and in a position outside the loop region. The present invention also provides composition in which the heterologous antigen is conjugated to the amino acid sequence. In preferred

15      embodiments, the heterologous antigen comprises at least one B cell epitope. In further preferred embodiments, the heterologous antigen comprises at least one T helper cell epitope. In exemplary embodiments, the heterologous antigen is chosen from but not limited to human immunodeficiency virus antigen, feline immunodeficiency virus antigen, *Plasmodium* parasite antigen, influenza virus antigen, *Staphylococcus* bacterium antigen, cholestryl ester transfer

20      protein antigen, major histocompatibility complex antigen, cytokine antigen, amyloid  $\beta$ -peptide antigen, peanut allergen antigen, latex allergen hevein antigen, brown shrimp allergen antigen and major grass pollen allergen antigen.

In some embodiments, the amino acid sequence further comprises at least one immune enhancer sequence. The immune enhancer sequence is operably linked to the C-terminus of the

25      amino acid sequence in some embodiments. In exemplary embodiments, the immune enhancer sequence is chosen from but not limited to unmethylated CpG dinucleotides, CD40 ligand, complement C3d fragment, B cell activating factor, and soluble gene product of lymphocyte activation gene-3. Also provided are embodiments further comprising human hepatitis B virus core antigen chosen from wild type human hepatitis B virus core antigen and modified human

30      hepatitis B virus core antigen lacking a heterologous antigen. In some embodiments, the composition further comprises one or more compounds chosen from adjuvant, diluent and

carrier. In related embodiments, the adjuvant is chosen from but not limited to aluminum hydroxide, aluminum phosphate, squalene and mineral oil. Moreover, the present invention provides a nucleic acid sequence encoding a heterologous antigen linked to the amino acid sequence set forth in SEQ ID NO:58. Related embodiments provide an expression vector 5 comprising a nucleic acid sequence encoding a heterologous antigen linked to the amino acid sequence set forth in SEQ ID NO:58.

Additionally, the present invention provides compositions comprising a heterologous antigen linked to an amino acid sequence which is at least 95% identical to the sequence set forth in SEQ ID NO:58, the amino acid sequence comprising a loop region and forming a particle 10 having a diameter of 25 to 35 nm. In some embodiments, the heterologous antigen comprises at least one modification chosen from insertion of at least one acidic amino acid residue, and substitution of at least one acidic amino acid residue. In some preferred embodiments, the at least one acidic amino acid residue comprises from 1 to 10 residues of one or both of aspartic acid and glutamic acid. In other preferred embodiments, the amino acid sequence comprises at 15 least one modification chosen from insertion of at least one acidic amino acid residue, and substitution of at least one acidic amino acid residue. In some preferred embodiments, the at least one acidic amino acid residue comprises from 1 to 10 residues of one or both of aspartic acid and glutamic acid.

Also provided by the present invention are compositions comprising the amino acid sequence set forth in SEQ ID NO:58, the amino acid sequence comprising a loop region and further comprising from 1 to 100 amino acids at the carboxy end of residue I<sup>149</sup>. In some preferred embodiments, the 1 to 100 amino acids is chosen from R<sup>150</sup>, K<sup>150</sup>, A<sup>150</sup>, R<sup>150</sup>R<sup>151</sup>C<sup>152</sup>, and SEQ ID NOS:3-6, 43-56 (excluding C<sup>150</sup>, and the wild type C-terminus set forth in SEQ ID NO:42). In other preferred embodiments, the 1 to 100 amino acids is chosen from SEQ ID 20 NOS:2, 7-20. In still further preferred embodiments, the 1 to 100 amino acids is chosen from SEQ ID NOS:22-36. Additionally, in particularly preferred embodiments, the heterologous antigen linked to the amino acid sequence set forth in SEQ ID NO:58, comprises a particle 25 having a diameter of 25 to 35 nm.

Additionally, in some embodiments, the amino acid sequence further comprises at least 30 one immune enhancer sequence. In preferred embodiments, the immune enhancer sequence is operably linked to the C-terminus of the amino acid sequence. In exemplary embodiments, the

immune enhancer sequence is chosen from but not limited to unmethylated CpG dinucleotides, CD40 ligand, complement C3d fragment, B cell activating factor, and soluble gene product of lymphocyte activation gene-3. Also provided is a nucleic acid sequence encoding the amino acid sequence set forth in SEQ ID NO: 58, the amino acid sequence comprising a loop region.

5 Related embodiments provide an expression vector comprising the nucleic acid sequence encoding the amino acid sequence set forth in SEQ ID NO:58. Additionally, compositions further comprising a modified human hepatitis B virus core antigen comprising a heterologous antigen are provided. In some particularly preferred embodiments, compositions are provided comprising an amino acid sequence which is at least 95% identical to SEQ ID NO:58, the amino acid sequence comprising a loop region and forming a particle having a diameter of 25 to 35 nm.

10

Importantly, the present invention provides methods, comprising: providing: a mammalian subject; and a composition comprising one or more of a polypeptide comprising a heterologous antigen linked to the amino acid sequence set forth in SEQ ID NO:58, the amino acid sequence comprising a loop region, and an expression vector encoding the polypeptide; and 15 administering the composition to the subject under conditions such that an immune response is generated. In some embodiments, the immune response comprises one or more of lymphocyte proliferative response, cytokine response and antibody response. In some preferred embodiments, the cytokine response comprises IL-2 production. In further embodiments, the antibody response comprises at least three fold higher levels of antibody than that observed 20 before administration of the at least one composition. In particularly preferred embodiments, the antibody response comprises production of IgG antibodies. In related embodiments, the IgG antibodies comprise an autoantibody. In some preferred embodiments, the composition further comprises one or more compounds chosen from adjuvant, diluent and carrier. In related embodiments, the adjuvant is chosen from but not limited to aluminum hydroxide, aluminum 25 phosphate, squalene and mineral oil.

Also provided by the present invention are methods for producing an immunogenic composition, comprising: providing: a heterologous antigen; and a hepatitis virus core antigen; altering at least one of the heterologous antigen and the hepatitis virus core antigen, with a modification chosen from insertion of at least one acidic amino acid residue and substitution of 30 at least one acidic amino acid residue; and inserting the heterologous antigen of step b within the hepatitis virus core antigen of step b to produce a modified hepatitis virus core antigen;

expressing the modified hepatitis virus core antigen under conditions suitable for producing particles having a diameter of 25 to 35 nm. In some embodiments, in the absence of the altering, expression of the modified hepatitis virus core antigen yields aggregates rather than particles. In other embodiments, in the absence of the altering, expression of the modified hepatitis virus core  
5 antigen yields 25 fold less particles than does expression of a wild type hepatitis virus core antigen. In some preferred embodiments, the at least one acidic amino acid residue comprises from 1 to 10 aspartic acid residues. In other preferred embodiments, the at least one acidic amino acid residue comprises from 1 to 10 glutamic acid residues. In related embodiments, the at least one acidic amino acid residue comprises at least one aspartic acid residue and at least one  
10 glutamic acid residue. In some embodiments, the insertion is in at least one position chosen from the N-terminus and the C-terminus of the heterologous antigen. In other embodiments, the substitution comprises a replacement of at least one non-acidic amino acid residue within the heterologous antigen, with the at least one acidic amino acid residue. In preferred embodiments, the altering produces a modified heterologous antigen with an isoelectric point in the range of 2.0  
15 to 7.0.. In a subset of these embodiments, the altering produces a modified heterologous antigen with an isoelectric point more preferably in the range of 3.0 to 6.0, and most preferably in the range of 4.0 to 5.0. . In a particularly preferred embodiment, the hepatitis virus core antigen is a human hepatitis B virus core antigen.

20           **DESCRIPTION OF THE FIGURES**

Figure 1 depicts the structure of the HBc particle determined at 7.4 angstrom resolution from cryoelectron micrographs. The immunodominant loop from amino acid residues 76 to 82 is shown.

Figure 2 provides a graph showing the comparative immunogenicity of HBcAg-based  
25 (HBc-M) and WHcAg-based (WHc-M) (NANP)<sub>n</sub> vaccines. Groups of three mice were immunized with a single dose of 20 µg of the indicated particles in IFA and sera were collected at the indicated time points for determination of anti-NANP titer by ELISA.

Figure 3 illustrates that the WHcAg accommodates insertion of foreign epitopes at many positions, with insertion sites indicated as numbered symbols. Tolerant (+) insertion sites are  
30 shown with an oval, intermediate (+/-) insertion sites are shown with a triangle, and non-tolerant (-) insertion sites are shown with a square.

Figure 4 provides an image of a 1% agarose gel indicating that wild type HBcAg and WHcAg particles migrate differently under non-denaturing conditions.

Figure 5 graphically depicts the magnitude of the antibody response elicited by immunizing mice of the indicated H-2 congenic strains with 7.0 µg of either WHcAg or HBcAg in IFA. Six weeks post immunization (1°, 6wk), sera were collected and analyzed for anti-WHc (solid) or anti-HBc (hatched) antibody by ELISA. End-point serum titers are shown.

Figure 6 illustrates that there is a low level of cross-reactivity between the WHcAg and the HBcAg at the antibody level. The indicated panel of H-2 congenic strains was immunized with 7.0 µg of either WHcAg (solid) or HBcAg (hatched) in IFA. Six weeks later sera were collected and tested for binding to both WHcAg and HBcAg. End-point serum titers are shown.

Figure 7 illustrates that there is a low level of cross-reactivity between the WHcAg and the HBcAg at the T cell level. Balb/c mice were immunized with WHcAg (5.0 µg) in complete Freund's adjuvant. Ten days later draining lymph node (LN) cells were harvested and cultured with varying concentrations of WHcAg and HBcAg *in vitro*. T cell proliferation was measured by [<sup>3</sup>H]TdR uptake and corrected for background.

Figure 8 shows the proliferative response of cells from mice of the indicated strains and H-2 haplotypes after immunization with WHcAg (5.0 µg) in CFA. Ten days later draining LN cells were cultured with WHcAg, HBcAg and the indicated peptides *in vitro*, and T cell proliferation was measured by [<sup>3</sup>H]TdR uptake. Panel A shows the proliferative response of B10.S mice to WHc, HBc and the Wp120-131 (VSFGVWIRTPAP, set forth as SEQ ID NO:59; while the corresponding HBV sequence is VSFGVWIRTPPA, set forth as SEQ ID NO:60). Panel B shows the proliferative response of cells of B10.D2 mice to WHc, HBc, and the Wp60-80 peptide (VCWDELTKLIAWMSSNITSEQ, set forth as SEQ ID NO:61; while the corresponding HBV sequence is LCWGELMTLATWVGGNLEDPI, set forth as SEQ ID NO:62). Panel C shows the proliferative response of cells from B10.M mice to WHc, HBc, and the Wp60-80 peptide. The peptide T cell site recognized after HBcAg immunization of B10.S mice is Hp120-131, of B10.D2 mice is Hp85-100, and of B10.M mice is Hp100-120.

Figure 9 provides the results of a CD4+ T cell epitope mapping analysis in the indicated strains of mice. Briefly, mice were immunized (7.0 µg) and boosted (3.5 µg) with either WHcAg or HBcAg in IFA, and ten days later spleen cells were cultured with WHcAg, HBcAg and a panel of WHcAg-derived or HBcAg-derived peptides *in vitro*. After 4 days, tissue culture

supernatants (SN) were collected and IFN $\gamma$  ( $\mu$ g/ml) was measured by ELISA. The relative strengths of the peptide T cell sites are indicated by the minimum peptide concentrations required to recall IFN $\gamma$  production *in vitro*.

Figure 10 provides the results of a CD4+ T cell epitope mapping analysis in the indicated strains of mice. Briefly, mice were immunized (7.0  $\mu$ g) and boosted (3.5  $\mu$ g) with either WHcAg or HBcAg in IFA, and ten days later spleen cells were cultured with WHcAg, HBcAg and a panel of WHcAg-derived or HBcAg-derived peptides *in vitro*. After 4 days, tissue culture supernatants (SN) were collected and IFN $\gamma$  ( $\mu$ g/ml) was measured by ELISA. The relative strengths of the peptide T cell sites are indicated by the minimum peptide concentrations required to recall IFN $\gamma$  production *in vitro*.

Figure 11 illustrates that anti-WHc antibodies do not interfere with the commercial anti-HBc diagnostic assay. The commercial anti-HBc assay was performed as recommended by the supplier with the exception that 1:10 dilutions of murine sera were used.

Figure 12, panels A-E, illustrate that WHc is an effective immunogen in both wild type and transgenic mice. Wild type (+/+) and HBeAg-expressing transgenic (HBe-Tg) mice were immunized with 20  $\mu$ g of either HBcAg, an HBc-based malaria vaccine candidate (HBc-M, V12.PF3.1) or an WHc-based malaria vaccine candidate (WHc-M, HyW-M78) in IFA. Sera were collected at 2, 4 and 8 weeks after immunization and analyzed for anti-core antibody (left panels A, B, and C) and anti-NANP antibody (right panels D and E) by ELISA.

Figure 13, panels A-F, depict the recall IFN $\gamma$ -response of wild type and HBe-Tg mice after immunizing with 10  $\mu$ g of HBcAg (panels A and D), HBc-M (panels B and E) or WHc-M (panels C and F) as determined by ELISA of four day culture SNs. Two weeks later spleen cells were collected and cultured with HBc, WHc and p120-140 peptide, conserved between WHc and HBc (W/H), or the WHc-derived peptides p60-80 (W) and p80-95 (W), which are not conserved.

Figures 14 and 15 show the immunogenicity of hybrid core particles. The indicated hybrid particles were injected (20  $\mu$ g, 1<sup>o</sup>) and boosted (10  $\mu$ g, 2<sup>o</sup>) in IFA. Eight weeks after the primary and 6 weeks after the secondary immunization, sera were collected, pooled, and anti-insert and anti-core antibody titers were determined by ELISA. The hybrid particles are designated by the C-terminus, the inserted epitope (e.g., M = malaria *P. falciparum*) and the position of the insert (e.g., 74). Full descriptions of the C-termini and the inserted epitopes are listed in the accompanying tables.

Figure 16 shows the effect of the position of the inserted epitope (NANP)<sub>n</sub> on the immunogenicity of the WHcAg hybrid particle. Groups of 4 mice were primed with 20 µg of the indicated particles in IFA. Sera were collected, pooled, and analyzed for anti-NANP by ELISA. The hybrid particles are identical except for the position of the insert: NH<sub>2</sub>-terminus, COOH-terminus, or internal (e.g., amino acid 78 or 74).

Figure 17 illustrates the effect on immunogenicity of varying the C-termini of hybrid WHcAg particles with the same (M) heterologous sequence inserted at amino acid 74. In this study, mice were immunized with a single dose of 20 µg of the hybrid particles in IFA.

Figure 18 graphically depicts the lack of genetic nonresponders and the magnitude of the antibody response elicited by immunizing mice of the indicated H-2 congenic strains with 10 µg of the WHc-based malaria vaccine candidate (HyW-M78) in IFA and boosting with 5.0 µg of HyW-M78 in IFA. Six weeks after the primary (1<sup>o</sup>) and the secondary (2<sup>o</sup>) immunizations, sera were collected and anti-WHc and anti-NANP antibodies were determined by ELISA.

Figure 19 provides the results of a CD4+ T cell epitope mapping analysis in the indicated strains of mice. Briefly, mice of the indicated strains and H-2 haplotypes were immunized and boosted either with WHcAg (7.0 µg) or a WHc-based malaria vaccine candidate (HyW-M78) (10 µg) both in IFA. Two weeks after the boost spleen cells were harvested and cultured with a panel of WHcAg-derived peptides *in vitro*. Four day culture SNs were collected and IFN $\gamma$  was measured by ELISA. The minimum concentration of peptide required to recall IFN $\gamma$  production is indicated by the shaded boxes representing weak (light) to very strong (dark) T cell sites.

Figure 20 provides a schematic representation of the steps involved in construction of the modified WHcAg vaccine platform.

Figure 21 depicts the results of capture ELISAs designed to detect either WHcAg polypeptide as a marker for expression or WHcAg particles as a marker for assembly in *E. coli* lysates. In panel A, a polyclonal antibody that recognizes only assembled particles (anti-nWHc) is used to determine relative assembly competence, while in panel B, a mAb specific for a peptidic epitope on WHcAg (anti-pWHc) is used as the detecting antibody to determine relative expression levels. In addition, a malaria (M) epitope-specific mAb was used to detect the malaria repeat epitope (dashed line). The capture antibody does not compete with either detecting antibody.

Figure 22 provides a list of WHcAg-IM2 or WHcAg -IM2 mutant(-) hybrid constructs expressed in *E. coli* and analyzed for relative expression level and assembly competence by capture ELISA. Lysates were sequentially screened with mAbs that preferentially recognize denatured WHcAg or assembled WHcAg particles and given relative scores accordingly.

5 Figure 23 shows an analysis of a M2e peptide analog panel for binding to mAb 14C2 and to a polyclonal murine anti-HyW-IM2(-)78 antisera. The wild type M2e sequence is set forth herein as SEQ ID NO:64.

10 Figure 24 illustrates that particulate HBcAg is preferentially presented by naive B cells to naive T cells. Either splenic adherent cells including macrophage and dendritic cells (MØ/DC) or B cells from naive mice were fractionated and used as APC for fractionated CD4<sup>+</sup> T cells derived from naive TCR-Tg (core-specific) mice. Purified APC plus CD4<sup>+</sup> T cells were cultured in the presence of HBcAg or peptide for 48 hrs before the level of IL-2 in the SN was determined by ELISA.

15 Figure 25 illustrates that particulate WHcAg and hybrid WHcAg particles are preferentially presented by naive B cells to naive T cells. Either naive spleen cells from wild type mice or from B cell knockout (KO) mice were used as APCs for CD4<sup>+</sup> T cells derived from core-specific TCR-Tg mice. Purified APC plus CD4<sup>+</sup> T cells were cultured in the presence of the indicated antigen for 48 hrs before the level of IL-2 in the SN was determined by ELISA.

20 Figure 26 shows the magnitude of the *in vitro* primary antibody response elicited by HBc, HBe, and WHc. Briefly, spleen cells derived from core-specific TCR-Tg mice were cultured for 5 days in the presence of the indicated antigen before SNs were collected and analyzed for the respective IgM antibodies by ELISA.

25 Figure 27 illustrates the correlation observed between *in vivo* anti-insert IgG antibody production and primary *in vitro* IgM antibody production. *In vitro* IgM was determined by ELISA, using the respective hybrid particles as solid phase ligands, while the *in vivo* anti-insert IgG level was measured on solid phase peptides.

Figure 28 provides a schematic representation of one of the methods of the present invention used to obtain mosaic WHcAg particles by the read-through mechanism.

30 Figure 29 provides a schematic representation of one of the methods of the present invention used to obtain mosaic WHcAg particles by utilization of differentially induced plasmids.

Figure 30 shows the effect adjuvant usage on the level of insert-specific antibody production. Groups of mice were immunized with C-longM78 hybrid WHcAg particles in saline (1<sup>o</sup>; primary and 2<sup>o</sup>, secondary) or formulated in the indicated adjuvants. Sera were collected at 6 weeks post immunization; pooled and analyzed for anti-NANP antibody by ELISA, as shown 5 in Panel A. Panel B depicts the IgG isotype distribution pattern of anti-NANP antibodies elicited by C-longM78 particles administered in saline or the indicated adjuvants.

Figure 31 shows that antibodies are raised to a protein which is chemically-coupled to WHcAg. Balb/c mice were immunized three times with a WHcAg-PGA chemical conjugate (10 µg) adsorbed on alum. Two weeks after each immunization sera were collected and IgM and 10 IgG anti-PGA antibodies and anti-WHc antibody was determined by ELISA.

Figure 32 depicts the antibody response over the course of four months after immunization of mice with an Influenza A M2e-WHcAg hybrid particle (HyW-IM2(-)78). Five mice were immunized with 20 µg (1<sup>o</sup>) and boosted with 10 µg (2<sup>o</sup>) of M2e-WHcAg hybrid particles in IFA, and sera was collected, pooled and analyzed for anti-WHc and anti-M2e 15 antibodies by ELISA.

Figure 33 shows the IgG isotype distribution of primary (1<sup>o</sup>) and secondary (2<sup>o</sup>) sera reactive with M2e (panel A) and WHc (panel B) of mice immunized with M2e-WHcAg hybrid particles as described in Figure 33.

Figure 34 illustrates that sera from mice immunized with WHcAg-M2e reacts with 20 influenza A virus infected cells. Sera from WHcAg immunized (panel A) or WHcAg-M2e (panel B) immunized mice were incubated with mock (open histograms) or influenza A-infected (filled histograms) 293T cells. After incubation with a goat anti-mouse IgG conjugated to FITC, the cells were analyzed by flow cytometry.

Figure 35 depicts the inhibition of rWSN M-Udorn replication by sera from WHcAg- 25 M2e immunized mice. MDCK cells were infected at an MOI of 0.1 for 1 hour, with either rWSN (anti-M2e insensitive) (open bars) or the anti-M2e sensitive, rWSN M-Udorn (hatched bars) strain. Cells were washed extensively then incubated in DMEM containing trypsin and 1% sera from WHcAg-M2e immunized mice. At the indicated times post infection supernatants were collected and infectious virus particle concentration determined by plaque assay.

Figure 36 depicts the antibody response obtained upon immunization (20 µg) and 30 boosting (10 µg) (B10 x B10.S)<sub>F1</sub> mice with hybrid WHcAg particles containing a CETP<sub>461-476</sub>

insert (HyW-CE<sub>74</sub>) in IFA. Sera was collected at the indicated times and anti-WHc and anti-CETP<sub>461-476</sub> was determined by ELISA. The 14 week sera was tested for the ability to inhibit human CETP enzymatic activity *in vitro* (inset). The human CETP was obtained from hCETP-Tg mouse sera.

5       Figure 37 illustrates the *in vivo* persistence of induced autoantibody. Double-Tg mice expressing an HBc/HBe-specific TCR (7/16-5) and either HBeAg or HBcAg were injected with the TCR target peptide (aa 129-140) at day 0. Sera were collected at the indicated times and anti-HBe (panel A) and anti-HBc autoantibody (panel B) was determined by ELISA.

10      Figure 38 depicts the migration patterns of WHcAg and WHcAg-CD40L particles in a polyacrylamide gel following denaturing and reducing conditions (SDS plus beta-mercaptoethanol). The amino acid sequence of the WHcAg-CD40L is set forth herein as SEQ ID NO:69. The predicted molecular weights of the two core proteins are shown: WHc travels as a 20.5 kDa monomer and a 41.1 kDa dimer; and WHc-CD40L travels as a 37.4 kDa monomer.

15      Figure 39 illustrates that the addition of the molecular adjuvant CD40L enhances the immunogenicity of WHcAg. Mice were immunized with 20 µg of WHcAg particles or WHcAg-CD40L hybrid particles in IFA. At one week (Panel A) and two weeks (Panel B) post-immunization, sera were collected and analyzed for anti-WHc antibody by ELISA.

20      Figure 40 provides the wild type WHcAg nucleic acid sequence (Panel A), and the amino acid sequences of both wild type WHcAg (Panel B) and truncated WHcAg (Panel C), as set forth in SEQ ID NO:37, SEQ ID NO:1, and SEQ ID NO:38 respectively.

Figure 41 provides the wild type GSHcAg nucleic acid sequence (Panel A), and the amino acid sequences of both wild type GSHcAg (Panel B) and truncated GSHcAg (Panel C), as set forth in SEQ ID NO:39, SEQ ID NO:21, and SEQ ID NO:40 respectively.

25      Figure 42 provides the wild type HBcAg nucleic acid sequence (Panel A), and the amino acid sequences of both wild type HBcAg (Panel B) and truncated HBcAg (Panel C), as set forth in SEQ ID NO:57, SEQ ID NO:41, and SEQ ID NO:58 respectively.

#### GENERAL DESCRIPTION OF THE INVENTION

The present invention is directed to exploitation of hepadna virus nucleocapsids/core antigens as multivalent carrier platforms for enhancing the immune response to weak haptenic-like antigens. During development of the present invention, the theoretical and practical

limitations inherent to the original human hepatitis B virus (HBV) nucleocapsid/core antigen (HBcAg) platform technology were addressed. In one embodiment, a new combinatorial platform technology was developed by modification of the woodchuck hepadna virus (WHV) core antigen (WHcAg). To begin with, three variables were identified as considerations in

5     designing WHcAg-hybrid particles: the insert position, the C-terminal sequence and the epitope sequence. A rapid screening method to examine WHcAg-hybrid particle assembly within bacterial lysates was developed as part of a combinatorial approach involving shuffling of the insert position, and C-terminal modifications for each epitope of interest. In another embodiment, a second new combinatorial platform technology is developed by modification of

10    the ground squirrel hepadna virus (GHV) nucleocapsid/core antigen (GSHcAg). While in a further embodiment, the human hepatitis B virus core antigen platform is improved through introduction of various modifications.

#### DEFINITIONS

15    The term "gene" refers to a nucleic acid (e.g., DNA) sequence that comprises coding sequences necessary for the production of a polypeptide or precursor or RNA (e.g., tRNA, siRNA, rRNA, etc.). The polypeptide can be encoded by a full length coding sequence or by any portion of the coding sequence so long as the desired activity or functional properties (e.g., enzymatic activity, ligand binding, signal transduction, etc.) of the full-length or fragment are

20    retained. The term also encompasses the coding region of a structural gene and the sequences located adjacent to the coding region on both the 5' and 3' ends, such that the gene corresponds to the length of the full-length mRNA. The sequences that are located 5' of the coding region and which are present on the mRNA are referred to as 5' untranslated sequences. The sequences that are located 3' or downstream of the coding region and that are present on the mRNA are referred

25    to as 3' untranslated sequences. The term "gene" encompasses both cDNA and genomic forms of a gene. A genomic form or clone of a gene contains the coding region, which may be interrupted with non-coding sequences termed "introns" or "intervening regions" or "intervening sequences." Introns are removed or "spliced out" from the nuclear or primary transcript, and are therefore absent in the messenger RNA (mRNA) transcript. The mRNA functions during translation to

30    specify the sequence or order of amino acids in a nascent polypeptide.

In particular, the term "WHcAg gene" refers to the full-length WHcAg nucleotide sequence (e.g., contained in SEQ ID NO:37). However, it is also intended that the term encompass fragments of the WHcAg sequence, and/or other domains within the full-length WHcAg nucleotide sequence. Furthermore, the terms "WHcAg nucleotide sequence" or "WHcAg polynucleotide sequence" encompasses DNA, cDNA, and RNA (e.g., mRNA) sequences.

The term "plasmid" as used herein, refers to a small, independently replicating, piece of DNA. Similarly, the term "naked plasmid" refers to plasmid DNA devoid of extraneous material typically used to affect transfection. As used herein, a "naked plasmid" refers to a plasmid substantially free of calcium-phosphate, DEAE-dextran, liposomes, and/or polyamines. As used herein, the term "purified" refers to molecules (polynucleotides or polypeptides) that are removed from their natural environment, isolated or separated. "Purified" molecules are at least 50% free, preferably at least 75% free, and more preferably at least 90% free from other components with which they are naturally associated.

The term "recombinant DNA" refers to a DNA molecule that is comprised of segments of DNA joined together by means of molecular biology techniques. Similarly, the term "recombinant protein" refers to a protein molecule that is expressed from recombinant DNA.

The term "fusion protein" as used herein refers to a protein formed by expression of a hybrid gene made by combining two gene sequences. Typically this is accomplished by cloning a cDNA into an expression vector in frame with an existing gene. The fusion partner may act as a reporter (e.g.,  $\beta$ gal) or may provide a tool for isolation purposes (e.g., GST).

Suitable systems for production of recombinant proteins include but are not limited to prokaryotic (e.g., *Escherichia coli*), yeast (e.g., *Saccharomyces cerevisiae*), insect (e.g., baculovirus), mammalian (e.g., Chinese hamster ovary), plant (e.g., safflower), and cell-free systems (e.g., rabbit reticulocyte).

As used herein, the term "coding region" refers to the nucleotide sequences that encode the amino acid sequences found in the nascent polypeptide as a result of translation of an mRNA molecule. The coding region is bounded in eukaryotes, on the 5' side by the nucleotide triplet "ATG" that encodes the initiator methionine and on the 3' side by one of the three triplets which specify stop codons (*i.e.*, TAA, TAG, and TGA).

Where amino acid sequence is recited herein to refer to an amino acid sequence of a protein molecule, "amino acid sequence" and like terms, such as "polypeptide" or "protein" are not meant to limit the amino acid sequence to the complete, native amino acid sequence associated with the recited protein molecule. Rather the terms "amino acid sequence" and "protein" encompass partial sequences, and modified sequences.

The term "wild type" refers to a gene or gene product that has the characteristics of that gene or gene product when isolated from a naturally occurring source. A wild type gene is that which is most frequently observed in a population and is thus arbitrarily designed the "normal" or "wild-type" form of the gene.

In contrast, the terms "modified," "mutant," and "variant" refer to a gene or gene product that displays modifications in sequence and or functional properties (*i.e.*, altered characteristics) when compared to the wild-type gene or gene product. In some embodiments, the modification comprises at least one insertion, deletion, or substitution. In preferred embodiments, the insertion comprises introduction of a heterologous antigen sequence into a hepatitis B virus antigen sequence (*e.g.*, fusion protein).

The term "homology" refers to a degree of complementarity. There may be partial homology or complete homology (*i.e.*, identity). A partially complementary sequence is one that at least partially inhibits a completely complementary sequence from hybridizing to a target nucleic acid and is referred to using the functional term "substantially homologous." The term "inhibition of binding," when used in reference to nucleic acid binding, refers to reduction in binding caused by competition of homologous sequences for binding to a target sequence. The inhibition of hybridization of the completely complementary sequence to the target sequence may be examined using a hybridization assay (Southern or Northern blot, solution hybridization and the like) under conditions of low stringency. A substantially homologous sequence or probe will compete for and inhibit the binding (*i.e.*, the hybridization) of a completely homologous sequence to a target under conditions of low stringency. This is not to say that conditions of low stringency are such that non-specific binding is permitted; low stringency conditions require that the binding of two sequences to one another be a specific (*i.e.*, selective) interaction. The absence of non-specific binding may be tested by the use of a second target that lacks even a partial degree of complementarity (*e.g.*, less than about 30% identity); in the absence of non-specific binding the probe will not hybridize to the second non-complementary target.

The art knows well that numerous equivalent conditions may be employed to comprise low stringency conditions; factors such as the length and nature (DNA, RNA, base composition) of the probe and nature of the target (DNA, RNA, base composition, present in solution or immobilized, etc.) and the concentration of the salts and other components (e.g., the presence or 5 absence of formamide, dextran sulfate, polyethylene glycol) are considered and the hybridization solution may be varied to generate conditions of low stringency hybridization different from, but equivalent to, the above listed conditions. In addition, the art knows conditions that promote hybridization under conditions of high stringency (e.g., increasing the temperature of the hybridization and/or wash steps, the use of formamide in the hybridization solution, etc.).

10 When used in reference to a double-stranded nucleic acid sequence such as a cDNA or genomic clone, the term "substantially homologous" refers to any probe that can hybridize to either or both strands of the double-stranded nucleic acid sequence under conditions of low stringency as described above.

When used in reference to a single-stranded nucleic acid sequence, the term 15 "substantially homologous" refers to any probe that can hybridize (*i.e.*, it is the complement of) the single-stranded nucleic acid sequence under conditions of low stringency as described above.

As used herein, the term "competes for binding" when used in reference to a first and a second polypeptide means that the first polypeptide with an activity binds to the same substrate as does the second polypeptide with an activity. In one embodiment, the second polypeptide is a 20 variant of the first polypeptide (e.g., encoded by a different allele) or a related (e.g., encoded by a homolog) or dissimilar (e.g., encoded by a second gene having no apparent relationship to the first gene) polypeptide. The efficiency (e.g., kinetics or thermodynamics) of binding by the first polypeptide may be the same as or greater than or less than the efficiency of substrate binding by the second polypeptide. For example, the equilibrium binding constant ( $K_D$ ) for binding to the 25 substrate may be different for the two polypeptides. The term " $K_m$ " as used herein refers to the Michaelis-Menton constant for an enzyme and is defined as the concentration of the specific substrate at which a given enzyme yields one-half its maximum velocity in an enzyme catalyzed reaction.

As used herein, the term "hybridization" is used in reference to the pairing of 30 complementary nucleic acids. Hybridization and the strength of hybridization (*i.e.*, the strength of the association between the nucleic acids) is impacted by such factors as the degree of

complementary between the nucleic acids, stringency of the conditions involved, the  $T_m$  of the formed hybrid, and the G:C ratio within the nucleic acids.

As used herein, the term " $T_m$ " is used in reference to the "melting temperature." The melting temperature is the temperature at which a population of double-stranded nucleic acid molecules becomes half dissociated into single strands. The equation for calculating the  $T_m$  of nucleic acids is well known in the art. As indicated by standard references, a simple estimate of the  $T_m$  value may be calculated by the equation:  $T_m = 81.5 + 0.41(\% G + C)$ , when a nucleic acid is in aqueous solution at 1 M NaCl (See e.g., Anderson and Young, Quantitative Filter Hybridization, in *Nucleic Acid Hybridization* [1985]). Other references include more sophisticated computations that take structural as well as sequence characteristics into account for the calculation of  $T_m$ .

As used herein the term "stringency" is used in reference to the conditions of temperature, ionic strength, and the presence of other compounds such as organic solvents, under which nucleic acid hybridizations are conducted. Those skilled in the art will recognize that "stringency" conditions may be altered by varying the parameters just described either individually or in concert. With "high stringency" conditions, nucleic acid base pairing will occur only between nucleic acid fragments that have a high frequency of complementary base sequences (e.g., hybridization under "high stringency" conditions may occur between homologs with 85-100% identity, preferably 70-100% identity). With medium stringency conditions, nucleic acid base pairing will occur between nucleic acids with an intermediate frequency of complementary base sequences (e.g., hybridization under "medium stringency" conditions may occur between homologs with 50-70% identity). Thus, conditions of "weak" or "low" stringency are often required with nucleic acids that are derived from organisms that are genetically diverse, as the frequency of complementary sequences is usually less.

"High stringency conditions" when used in reference to nucleic acid hybridization comprise conditions equivalent to binding or hybridization at 42 °C in a solution comprising 5X SSPE (43.8 g/l NaCl, 6.9 g/l NaH<sub>2</sub>PO<sub>4</sub> H<sub>2</sub>O and 1.85 g/l EDTA, pH adjusted to 7.4 with NaOH), 0.5% SDS, 5X Denhardt's reagent and 100 µg/ml denatured salmon sperm DNA followed by washing in a solution comprising 0.1X SSPE, 1.0% SDS at 42 °C when a probe of about 100 to about 1000 nucleotides in length is employed.

"Medium stringency conditions" when used in reference to nucleic acid hybridization comprise conditions equivalent to binding or hybridization at 42 °C in a solution comprising 5X SSPE (43.8 g/l NaCl, 6.9 g/l NaH<sub>2</sub>PO<sub>4</sub> H<sub>2</sub>O and 1.85 g/l EDTA, pH adjusted to 7.4 with NaOH), 0.5% SDS, 5X Denhardt's reagent and 100 µg/ml denatured salmon sperm DNA 5 followed by washing in a solution comprising 1.0X SSPE, 1.0% SDS at 42 °C when a probe of about 100 to about 1000 nucleotides in length is employed.

"Low stringency conditions" comprise conditions equivalent to binding or hybridization at 42 °C in a solution comprising 5X SSPE (43.8 g/l NaCl, 6.9 g/l NaH<sub>2</sub>PO<sub>4</sub> H<sub>2</sub>O and 1.85 g/l EDTA, pH adjusted to 7.4 with NaOH), 0.1% SDS, 5X Denhardt's reagent [50X Denhardt's 10 contains per 500 ml: 5 g Ficoll (Type 400, Pharamcia), 5 g BSA (Fraction V; Sigma)] and 100 g/ml denatured salmon sperm DNA followed by washing in a solution comprising 5X SSPE, 0.1% SDS at 42 °C when a probe of about 100 to about 1000 nucleotides in length is employed.

The term "equivalent" when made in reference to a hybridization condition as it relates to a hybridization condition of interest means that the hybridization condition and the hybridization 15 condition of interest result in hybridization of nucleic acid sequences which have the same range of percent (%) homology. For example, if a hybridization condition of interest results in hybridization of a first nucleic acid sequence with other nucleic acid sequences that have from 85% to 95% homology to the first nucleic acid sequence, then another hybridization condition is said to be equivalent to the hybridization condition of interest if this other hybridization 20 condition also results in hybridization of the first nucleic acid sequence with the other nucleic acid sequences that have from 85% to 95% homology to the first nucleic acid sequence.

The following terms are used to describe the sequence relationships between two or more polynucleotides: "reference sequence", "sequence identity", "percentage of sequence identity", and "substantial identity". A "reference sequence" is a defined sequence used as a basis for a 25 sequence comparison; a reference sequence may be a subset of a larger sequence, for example, as a segment of a full-length cDNA sequence given in a sequence listing or may comprise a complete gene sequence. Generally, a reference sequence is at least 20 nucleotides in length, frequently at least 25 nucleotides in length, and often at least 50 nucleotides in length. Since two polynucleotides may each (1) comprise a sequence (*i.e.*, a portion of the complete polynucleotide 30 sequence) that is similar between the two polynucleotides, and (2) may further comprise a sequence that is divergent between the two polynucleotides, sequence comparisons between two

(or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity. A "comparison window", as used herein, refers to a conceptual segment of at least 20 contiguous nucleotide positions wherein a polynucleotide sequence may be compared to a reference sequence of at least 20 contiguous nucleotides and wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) of 20 percent or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith and Waterman (Smith and Waterman, *Adv. Appl. Math.*, 2: 482, 1981) by the homology alignment algorithm of Needleman and Wunsch (Needleman and Wunsch, *J. Mol. Biol.*, 48:443, 1970), by the search for similarity method of Pearson and Lipman (Pearson and Lipman, *Proc. Natl. Acad. Sci., U.S.A.*, 85:2444, 1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, Madison, WI), or by inspection, and the best alignment (i.e., resulting in the highest percentage of homology over the comparison window) generated by the various methods is selected. The term "sequence identity" means that two polynucleotide sequences are identical (i.e., on a nucleotide-by-nucleotide basis) over the window of comparison. The term "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, U, or I) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. The terms "substantial identity" as used herein denotes a characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence that has at least 85 percent sequence identity, preferably at least 90 to 95 percent sequence identity, more usually at least 99 percent sequence identity as compared to a reference sequence over a comparison window of at least 20 nucleotide positions, frequently over a window of at least 25-50 nucleotides, wherein the percentage of sequence identity is calculated by comparing the reference sequence to the polynucleotide sequence which may include deletions or additions which total 20 percent or less of the reference sequence over

the window of comparison. The reference sequence may be a subset of a larger sequence, for example, as a segment of the full-length sequences of the compositions claimed in the present invention (e.g., WHcAg)

As applied to polypeptides, the term "substantial identity" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 80 percent sequence identity, preferably at least 90 percent sequence identity, more preferably at least 95 percent sequence identity or more (e.g., 99 percent sequence identity). Preferably, residue positions which are not identical differ by conservative amino acid substitutions. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having acidic side chains is glutamic acid and aspartic acid; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine.

"Amplification" is a special case of nucleic acid replication involving template specificity. It is to be contrasted with non-specific template replication (*i.e.*, replication that is template-dependent but not dependent on a specific template). Template specificity is here distinguished from fidelity of replication (*i.e.*, synthesis of the proper polynucleotide sequence) and nucleotide (ribo- or deoxyribo-) specificity. Template specificity is frequently described in terms of "target" specificity. Target sequences are "targets" in the sense that they are sought to be sorted out from other nucleic acid. Amplification techniques have been designed primarily for this sorting out.

Template specificity is achieved in most amplification techniques by the choice of enzyme. Amplification enzymes are enzymes that, under conditions they are used, will process only specific sequences of nucleic acid in a heterogeneous mixture of nucleic acid. In particular, *Taq* and *Pfu* polymerases, by virtue of their ability to function at high temperature, are found to

display high specificity for the sequences bounded and thus defined by the primers; the high temperature results in thermodynamic conditions that favor primer hybridization with the target sequences and not hybridization with non-target sequences.

As used herein, the term "sample template" refers to nucleic acid originating from a sample that is analyzed for the presence of "target" (defined below). In contrast, "background template" is used in reference to nucleic acid other than sample template that may or may not be present in a sample. Background template is most often inadvertent. It may be the result of carryover, or it may be due to the presence of nucleic acid contaminants sought to be purified away from the sample. For example, nucleic acids from organisms other than those to be detected may be present as background in a test sample.

As used herein, the term "primer" refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product which is complementary to a nucleic acid strand is induced, (*i.e.*, in the presence of nucleotides and an inducing agent such as DNA polymerase and at a suitable temperature and pH). The primer is preferably single stranded for maximum efficiency in amplification, but may alternatively be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare extension products. Preferably, the primer is an oligodeoxyribonucleotide. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the inducing agent. The exact lengths of the primers will depend on many factors, including temperature, source of primer and the use of the method.

As used herein, the term "probe" refers to an oligonucleotide (*i.e.*, a sequence of nucleotides), whether occurring naturally as in a purified restriction digest or produced synthetically, recombinantly or by PCR amplification, that is capable of hybridizing to another oligonucleotide of interest. A probe may be single-stranded or double-stranded. Probes are useful in the detection, identification and isolation of particular WHcAg sequences. It is contemplated that any probe used in the present invention will be labelled with any "reporter molecule," so that is detectable in any detection system, including, but not limited to enzyme (*e.g.*, ELISA, as well as enzyme-based histochemical assays), fluorescent, radioactive, and

luminescent systems. It is not intended that the present invention be limited to any particular detection system or label.

As used herein, the term "target," when used in reference to the polymerase chain reaction, refers to the region of nucleic acid bounded by the primers used for polymerase chain reaction. Thus, the "target" is sought to be sorted out from other nucleic acid sequences. A "segment" is defined as a region of nucleic acid within the target sequence.

As used herein, the term "polymerase chain reaction" ("PCR") refers to the method of Mullis (U.S. Patent Nos. 4,683,195, 4,683,202, and 4,965,188, hereby incorporated by reference), that describe a method for increasing the concentration of a segment of a target sequence in a mixture of genomic DNA without cloning or purification. This process for amplifying the target sequence consists of introducing a large excess of two oligonucleotide primers to the DNA mixture containing the desired target sequence, followed by a precise sequence of thermal cycling in the presence of a DNA polymerase. The two primers are complementary to their respective strands of the double stranded target sequence. To effect amplification, the mixture is denatured and the primers then annealed to their complementary sequences within the target molecule. Following annealing, the primers are extended with a polymerase so as to form a new pair of complementary strands. The steps of denaturation, primer annealing, and polymerase extension can be repeated many times (*i.e.*, denaturation, annealing and extension constitute one "cycle"; there can be numerous "cycles") to obtain a high concentration of an amplified segment of the desired target sequence. The length of the amplified segment of the desired target sequence is determined by the relative positions of the primers with respect to each other, and therefore, this length is a controllable parameter. By virtue of the repeating aspect of the process, the method is referred to as the "polymerase chain reaction" (hereinafter "PCR"). Because the desired amplified segments of the target sequence become the predominant sequences (in terms of concentration) in the mixture, they are said to be "PCR amplified."

As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes, each of which cut double-stranded DNA at or near a specific nucleotide sequence.

The term "isolated" when used in relation to a nucleic acid, as in "an isolated oligonucleotide" or "isolated polynucleotide" refers to a nucleic acid sequence that is identified

and separated from at least one contaminant nucleic acid with which it is ordinarily associated in its natural source. Isolated nucleic acid is present in a form or setting that is different from that in which it is found in nature. In contrast, non-isolated nucleic acids are nucleic acids such as DNA and RNA found in the state they exist in nature. For example, a given DNA sequence

5 (e.g., a gene) is found on the host cell chromosome in proximity to neighboring genes; RNA sequences, such as a specific mRNA sequence encoding a specific protein, are found in the cell as a mixture with numerous other mRNAs that encode a multitude of proteins. However, isolated nucleic acid encoding gene includes, by way of example, such nucleic acid in cells

10 ordinarily expressing gene where the nucleic acid is in a chromosomal location different from that of natural cells, or is otherwise flanked by a different nucleic acid sequence than that found in nature. The isolated nucleic acid, oligonucleotide, or polynucleotide may be present in single-stranded or double-stranded form. When an isolated nucleic acid, oligonucleotide or polynucleotide is to be utilized to express a protein, the oligonucleotide or polynucleotide will

15 contain at a minimum the sense or coding strand (*i.e.*, the oligonucleotide or polynucleotide may be single-stranded), but may contain both the sense and anti-sense strands (*i.e.*, the oligonucleotide or polynucleotide may be double-stranded).

The terms "fragment" and "portion" when used in reference to a nucleotide sequence (as in "a portion of a given nucleotide sequence") refers to partial segments of that sequence. The fragments may range in size from four nucleotides to the entire nucleotide sequence minus one

20 nucleotide (10 nucleotides, 20, 30, 40, 50, 100, 200, etc.).

Similarly, the terms "fragment" and "portion" when used in reference to a polypeptide sequence refers to partial segments of that sequence. In some embodiments, the portion has an amino-terminal and/or carboxy-terminal deletion as compared to the native protein, but where the remaining amino acid sequence is identical to the corresponding positions in the amino acid

25 sequence deduced from a full-length cDNA sequence. Fragments are preferably at least 4 amino acids long, more preferably at least 50 amino acids long, and most preferably at least 50 amino acids long or longer (the entire amino acid sequence minus one amino acid). In particularly preferred embodiments, the portion comprises the amino acid residues required for intermolecular binding of the compositions of the present invention with its various ligands

30 and/or substrates.

As used herein the term "portion" when in reference to a protein (as in "a portion of a given protein") refers to fragments of that protein. The fragments may range in size from four consecutive amino acid residues to the entire amino acid sequence minus one amino acid.

5 As used herein the term "coding region" when used in reference to structural gene refers to the nucleotide sequences that encode the amino acids found in the nascent polypeptide as a result of translation of a mRNA molecule. The coding region is bounded, in eukaryotes, on the 5' side by the nucleotide triplet "ATG" that encodes the initiator methionine and on the 3' side by one of the three triplets which specify stop codons (*i.e.*, TAA, TAG, TGA).

10 As used herein, the term "purified" refers to molecules (polynucleotides or polypeptides) that are separated from other components with which they are naturally associated. "To purify" refers to a reduction (preferably by at least 10%, more preferably by at least 50%, and most preferably by at least 90%) of one or more contaminants from a sample. For example, WHcAg antibodies are purified by removal of contaminating non-immunoglobulin proteins; they are also purified by the removal of immunoglobulin that does not bind WHcAg. The removal of non-15 immunoglobulin proteins and/or the removal of immunoglobulins that do not bind WHcAg results in an increase in the percent of WHcAg-reactive immunoglobulins in the sample. In another example, recombinant WHcAg polypeptides are expressed in bacterial host cells and the polypeptides are purified by the removal of host cell proteins; the percent of recombinant WHcAg polypeptides is thereby increased in the sample.

20 The term "recombinant DNA molecule" as used herein refers to a DNA molecule that is comprised of segments of DNA joined together by means of molecular biological techniques. Similarly, the term "recombinant protein" or "recombinant polypeptide" as used herein refers to a protein molecule that is expressed from a recombinant DNA molecule.

25 The term "native protein" as used herein to indicate that a protein does not contain amino acid residues encoded by vector sequences, that is the native protein contains only those amino acids found in the protein as it occurs in nature. A native protein may be produced by recombinant means or may be isolated from a naturally occurring source.

30 The term "Southern blot," refers to the analysis of DNA on agarose or acrylamide gels to fractionate the DNA according to size followed by transfer of the DNA from the gel to a solid support, such as nitrocellulose or a nylon membrane. The immobilized DNA is then probed with a labeled probe to detect DNA species complementary to the probe used. The DNA may be

cleaved with restriction enzymes prior to electrophoresis. Following electrophoresis, the DNA may be partially depurinated and denatured prior to or during transfer to the solid support.

Southern blots are a standard tool of molecular biologists (Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, NY, pp 9.31-9.58, 1989).

5       The term "Northern blot," as used herein refers to the analysis of RNA by electrophoresis of RNA on agarose gels to fractionate the RNA according to size followed by transfer of the RNA from the gel to a solid support, such as nitrocellulose or a nylon membrane. The immobilized RNA is then probed with a labeled probe to detect RNA species complementary to the probe used. Northern blots are a standard tool of molecular biologists (Sambrook, *et al.*,

10      *supra*, pp 7.39-7.52, 1989).

The term "Western blot" refers to the analysis of protein(s) (or polypeptides) immobilized onto a support such as nitrocellulose or a membrane. The proteins are run on acrylamide gels to separate the proteins, followed by transfer of the protein from the gel to a solid support, such as nitrocellulose or a nylon membrane. The immobilized proteins are then exposed to antibodies with reactivity against an antigen of interest. The binding of the antibodies may be detected by various methods, including the use of radiolabelled antibodies.

15       The terms "antigenic determinant" and "epitope" as used herein refer to that portion of an antigen that makes contact with a particular antibody and/or T cell receptor. When a protein or fragment of a protein is used to immunize a host animal, numerous regions of the protein may induce the production of antibodies that bind specifically to a given region or three-dimensional structure on the protein; these regions or structures are referred to as antigenic determinants. An antigenic determinant may compete with the intact antigen (*i.e.*, the "immunogen" used to elicit the immune response) for binding to an antibody.

20       The term "transgene" as used herein refers to a foreign gene that is placed into an organism by introducing the foreign gene into newly fertilized eggs or early embryos. The term "foreign gene" refers to any nucleic acid (*e.g.*, gene sequence) that is introduced into the genome of an animal by experimental manipulations and may include gene sequences found in that animal so long as the introduced gene does not reside in the same location as does the naturally-occurring gene.

As used herein, the term "vector" is used in reference to nucleic acid molecules that transfer DNA segment(s) from one cell to another. The term "vehicle" is sometimes used interchangeably with "vector."

The term "expression vector" as used herein refers to a recombinant DNA molecule  
5 containing a desired coding sequence and appropriate nucleic acid sequences necessary for the expression of the operably linked coding sequence in a particular host organism. Nucleic acid sequences necessary for expression in prokaryotes usually include a promoter, an operator (optional), and a ribosome binding site, often along with other sequences. Eukaryotic cells are known to utilize promoters, enhancers, and termination and polyadenylation signals.

10 As used herein, the term host cell refers to any eukaryotic or prokaryotic cell (e.g., bacterial cells such as *E. coli*, yeast cells, mammalian cells, avian cells, amphibian cells, plant cells, fish cells, and insect cells), whether located *in vitro* or *in vivo*. For example, host cells may be located in a transgenic animal.

15 The term "transfection" as used herein refers to the introduction of foreign DNA into eukaryotic cells. Transfection may be accomplished by a variety of means known to the art including calcium phosphate-DNA co-precipitation, DEAE-dextran-mediated transfection, polybrene-mediated transfection, electroporation, microinjection, liposome fusion, lipofection, protoplast fusion, retroviral infection, and biolistics.

20 The term "stable transfection" or "stably transfected" refers to the introduction and integration of foreign DNA into the genome of the transfected cell. The term "stable transfectant" refers to a cell that has stably integrated foreign DNA into the genomic DNA.

25 The term "transient transfection" or "transiently transfected" refers to the introduction of foreign DNA into a cell where the foreign DNA fails to integrate into the genome of the transfected cell. The foreign DNA persists in the nucleus of the transfected cell for several days. During this time the foreign DNA is subject to the regulatory controls that govern the expression of endogenous genes in the chromosomes. The term "transient transfectant" refers to cells that have taken up foreign DNA but have failed to integrate this DNA.

30 The term "calcium phosphate co-precipitation" refers to a technique for the introduction of nucleic acids into a cell. The uptake of nucleic acids by cells is enhanced when the nucleic acid is presented as a calcium phosphate-nucleic acid co-precipitate. The original technique of is

modified to optimize conditions for particular types of cells. The art is well aware of these numerous modifications.

A "composition comprising a given polynucleotide sequence" as used herein refers broadly to any composition containing the given polynucleotide sequence. The composition may 5 comprise an aqueous solution. Compositions comprising polynucleotide sequences encoding WHcAg or fragments thereof may be employed as hybridization probes. In this case, the WHcAg encoding polynucleotide sequences are typically employed in an aqueous solution containing salts (e.g., NaCl), detergents (e.g., SDS), and other components (e.g., Denhardt's solution, dry milk, salmon sperm DNA, etc.).

10 As used herein, the terms "hepadnavirus" and "hepatitis virus" refer to any one of a family of DNA-containing viruses that cause hepatitis (inflammation of the liver) in a wide range of vertebrate species. The terms "hepatitis B virus" and "HBV" refers to a species of the genus orthohepadnavirus which causes human hepatitis B and which is also a causal agent in human hepatocellular carcinoma. Viruses similar to HBV also infect animals (e.g., woodchuck, ground 15 squirrel, duck), and are encompassed by some embodiments of the present invention.

The terms "WHcAg," "woodchuck hepadnavirus core antigen," and "woodchuck hepatitis virus core antigen" as used herein refer to the core antigen of the woodchuck hepadna virus exemplified by SEQ ID NO:1, while the WHcAg coding region is exemplified by SEQ ID NO:37. The term WHcAg also encompasses the core antigens of other woodchuck hepatitis 20 viruses, such as the woodchuck hepatitis virus clone 2 corresponding to GenBank Accession No. NK VLC2.

As used herein, the terms "GSHcAg," "ground squirrel hepadnavirus core antigen," and ground squirrel hepatitis virus core antigen" refer to the core antigen of the ground squirrel hepadna virus exemplified by SEQ ID NO:21, while the GSHcAg coding region is exemplified 25 by SEQ ID NO:39. The term GSHcAg also encompasses the core antigens of other ground squirrel hepadna viruses, such as the arctic ground squirrel hepatitis B virus corresponding to GenBank Accession No. NP\_040993.

The terms "HBcAg" and "human hepatitis B core antigen" refer to the core antigen of the human hepatitis B virus exemplified by SEQ ID NO:41, while the HBcAg coding region is 30 exemplified by SEQ ID NO:57. The term HBcAg also encompasses the core antigens of other HBV isolates, including but not limited to the ADW subtypes (e.g., subtype ADW4, strain brazil

/ isolate w4b; subtype ADW, strain okinawa/podw282; subtype ADW, strain indonesia/pidw420; etc.), and the ADR subtypes.

The term "hybrid" as used in reference to a hepadna virus core antigen, refers to a fusion protein of the hepadna virus core antigen and an unrelated antigen (e.g., Plasmodium antigen).

5 For instance, in some preferred embodiments of the present invention, the term "hybrid WHcAg" refers to a fusion protein comprising both a WHcAg component (full length, or partial) and a heterologous antigen (e.g., non-WHcAg and/or modified WHcAg) component. In particularly preferred embodiments, the heterologous antigen comprises at least one B cell epitope and/or at least one T cell epitope which may be conjugated (e.g., covalently linked) to a residue of the

10 WHcAg and/or which is inserted within the WHcAg via expression as a fusion protein. In contrast, the term "nonhybrid" refers to an antigen of a single origin (e.g., WHcAg in the absence of a heterologous antigen insert or conjugate).

The term "modified antigen" refers to an antigen, any part of which (such as the nucleic acid sequence and/or proteins) has been modified by chemical, biochemical, and/or molecular biological techniques compared to the wild-type antigen. In one embodiment, the antigen is modified by means of molecular biological techniques. In one embodiment, the modification may include one or more of a deletion, an insertion, and a substitution. A "deletion" is defined as a change in a nucleic acid sequence in which one or more nucleotides is absent. An "insertion" or "addition" is that change in a nucleic acid sequence which has resulted in the addition of one or more nucleotides. A "substitution" results from the replacement of one or more nucleotides by a one or more different nucleotides. Similarly, in some preferred embodiments, the term "modified WHcAg" refers to a woodchuck hepadna virus core protein with a C-terminal truncation. In particularly preferred embodiments, the modified WHcAg comprises both carboxy-terminal amino acid deletions, and insertions within the loop and/or outside the loop. In addition the term "modified WHcAg" refers to a woodchuck virus core protein comprising a heterologous antigen in the form of a conjugate or a fusion protein. Thus as used herein, the terms "modified hepatitis virus core antigen" and grammatical equivalents encompass hybrid core antigens, as well as mutant core antigens.

The term insertion of a first amino acid (e.g., alanine) or amino acid sequence (e.g., heterologous antigen) "at amino acid position x" or "in amino acid position x" of a second amino acid sequence (e.g., woodchuck hepadna virus core antigen) means introduction of a first amino

acid or sequence into a second amino acid sequence, such that the first amino acid or sequence is placed C-terminal to amino acid x.

The term "conjugating" when made in reference to two molecules (such as a heterologous antigen and hepadna virus core antigen) as used herein means covalently linking the two molecules. In one embodiment, where one of the molecules is a viral core or will be assembled into a viral nucleocapsid, it may be desirable to modify the nature and size of the second molecule and the site at which it is covalently linked to the core antigen such that it does not interfere with the capacity of the modified core to assemble *in vitro* and/or *in vivo*. In some embodiments, the heterologous antigen is conjugated to a functional group on the hepadna virus core antigen, chosen from but not limited to a carboxyl group, a primary amine, and a sulphydryl. In some preferred embodiments, a heterobifunctional cross-linker is used to attach the heterologous antigen to the hepadna virus core antigen. Exemplary cross-linkers include but are not limited to MBS, EDC/Sulfo-NHS and ABH obtained from Pierce (Rockford, IL).

As used herein in reference to a hepadna virus core antigen, the term "loop" refers to a portion of the hepadna virus core antigen which links the second and third alpha-helices and which contains an immunodominant B cell epitope. Specifically, in reference to HBcAG, the term "within the loop" refers to residues at positions 76 to 82 of the wild type sequence, while the term "outside the loop" refers to residues amino-terminal to residue 76 and carboxy-terminal to residue 82. Likewise, in reference to WHcAg, the term "within the loop" refers to residues at positions 76 to 82 of the wild type sequence, while the term "outside the loop" refers to residues amino-terminal to residue 76 and carboxy-terminal to residue 82. In contrast, in reference to GSHcAg, the term "within the loop" refers to residues at positions 76 to 81, while the term "outside the loop" refers to residues amino-terminal to residue 76 and carboxy-terminal to residue 81.

The terms "N-terminus" "NH<sub>2</sub>-terminus" and "amino-terminus" refer to the amino acid residue corresponding to the methionine encoded by the start codon (e.g., position or residue 1). In contrast the terms "C-terminus" "COOH-terminus" and "carboxy terminus" refer to the amino acid residue encoded by the final codon (e.g., last or final residue prior to the stop codon).

The term "antibody" refers to polyclonal and monoclonal antibodies. Polyclonal antibodies which are formed in the animal as the result of an immunological reaction against a protein of interest or a fragment thereof, can then be readily isolated from the blood using well-

known methods and purified by column chromatography, for example. Monoclonal antibodies can also be prepared using known methods (See, Winter and Milstein, *Nature*, 349, 293-299, 1991). As used herein, the term "antibody" encompasses recombinantly prepared, and modified antibodies and antigen-binding fragments thereof, such as chimeric antibodies, humanized 5 antibodies, multifunctional antibodies, bispecific or oligo-specific antibodies, single-stranded antibodies and F(ab) or F(ab)<sub>2</sub> fragments. The term "reactive" when used in reference to an antibody indicates that the antibody is capable of binding an antigen of interest. For example, a WHcAg-reactive antibody is an antibody which binds to WHcAg or to a fragment of WHcAg.

The terms "auto-antibody" or "auto-antibodies" refer to any immunoglobulin that binds 10 specifically to an antigen that is native to the host organism that produced the antibody (*i.e.*, the antigen is not synthetic and/or has not been artificially supplied to the host organism). However, the term encompasses antibodies originally produced in response to the administration or presence of a foreign and/or synthetic substance in the host, but also cross-react with "self" antigens. Exemplary auto-antibodies include, without limitation, anti-cholesterol ester transfer 15 protein (CETP) antibody, anti-major histocompatibility complex class II antibody, anti-cytokine antibody, and anti amyloid- $\beta$ -peptide antibody. The presence of auto-antibodies is termed "autoimmunity."

The term "cytokine" refers to a molecule, such a protein or glycoprotein, involved in the regulation of cellular proliferation and function. Cytokines are exemplified by lymphokines 20 (e.g., tumor necrosis factor- $\alpha$ , tumor necrosis factor- $\beta$ , interferon- $\gamma$ , *etc.*), growth-factors (e.g., erythropoietin, insulin, G-CSF, M-CSF, GM-CSF, EGF, PDGF, FGF, *etc.*), and interleukins (e.g., IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, *etc.*).

The term "B cell epitope" as used herein refers to as antigenic determinant (protein or carbohydrate) to which a single antibody molecule binds. B cell epitopes may comprise linear 25 epitopes (amino acids adjacent to each other in the primary sequence) or conformational epitopes (moieties distant from each other in the primary sequence, but which are brought in proximity to one another during folding of the antigen) of at least four amino acid residues.

The term "T cell epitope" as used herein refers to an antigenic determinant presented by a MHC class I or class II molecule for binding to a single T cell receptor. T cell epitopes are linear 30 epitopes comprising at least seven amino acid residues. In some embodiments of the present invention, the term T cell epitope comprises a T helper cell epitope which is an antigen fragment

presented by an MHC class II molecule for binding to T cell receptor on the surface of a helper T cell (e.g., generally CD4<sup>+</sup>).

The term "conservative substitution" as used herein refers to a change that takes place within a family of amino acids that are related in their side chains. Genetically encoded amino acids can be divided into four families: (1) acidic (aspartate, glutamate); (2) basic (lysine, arginine, histidine); (3) nonpolar (alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan); and (4) uncharged polar (glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine). Phenylalanine, tryptophan, and tyrosine are sometimes classified jointly as aromatic amino acids. In similar fashion, the amino acid repertoire can be grouped as (1) acidic (aspartate, glutamate); (2) basic (lysine, arginine, histidine), (3) aliphatic (glycine, alanine, valine, leucine, isoleucine, serine, threonine), with serine and threonine optionally being grouped separately as aliphatic-hydroxyl; (4) aromatic (phenylalanine, tyrosine, tryptophan); (5) amide (asparagine, glutamine); and (6) sulfur-containing (cysteine and methionine). Whether a change in the amino acid sequence of a peptide results in a functional homolog can be readily determined by assessing the ability of the variant peptide to function in a fashion similar to the wild-type protein. Peptides having more than one replacement can readily be tested in the same manner. In contrast, the term "nonconservative substitution" refers to a change in which an amino acid from one family is replaced with an amino acid from another family (e.g., replacement of a glycine with a tryptophan). Guidance in determining which amino acid residues can be substituted, inserted, or deleted without abolishing biological activity can be found using computer programs (e.g., LASERGENE software, DNASTAR Inc., Madison, WI).

The terms "antigen," "immunogen," "antigenic," "immunogenic," "antigenically active," and "immunologically active" refer to any substance that is capable of inducing a specific humoral and/or cell-mediated immune response. An immunogen generally contains at least one epitope. Immunogens are exemplified by, but not restricted to molecules which contain a peptide, polysaccharide, nucleic acid sequence, and/or lipid. Complexes of peptides with lipids, polysaccharides, or with nucleic acid sequences are also contemplated, including (without limitation) glycopeptide, lipopeptide, glycolipid, etc. These complexes are particularly useful immunogens where smaller molecules with few epitopes do not stimulate a satisfactory immune response by themselves.

A peptide sequence and nucleotide sequence may be "endogenous" or "heterologous" (i.e., "foreign"). The term "endogenous" refers to a sequence which is naturally found in the cell or virus into which it is introduced so long as it does not contain some modification relative to the naturally-occurring sequence. The term "heterologous" refers to a sequence which is not endogenous to the cell or virus into which it is introduced. For example, heterologous DNA includes a nucleotide sequence which is ligated to, or is manipulated to become ligated to, a nucleic acid sequence to which it is not ligated in nature, or to which it is ligated at a different location in nature. Heterologous DNA also includes a nucleotide sequence which is naturally found in the cell or virus into which it is introduced and which contains some modification relative to the naturally-occurring sequence. Generally, although not necessarily, heterologous DNA encodes heterologous RNA and heterologous proteins that are not normally produced by the cell or virus into which it is introduced. Examples of heterologous DNA include reporter genes, transcriptional and translational regulatory sequences, DNA sequences which encode selectable marker proteins (e.g., proteins which confer drug resistance), etc. In preferred embodiments, the terms "heterologous antigen" and "heterologous sequence" refer to a non-hepadna virus antigen or amino acid sequence including but not limited to microbial antigens, mammalian antigens and allergen antigens.

The terms "peptide," "peptide sequence," "amino acid sequence," "polypeptide," and "polypeptide sequence" are used interchangeably herein to refer to at least two amino acids or amino acid analogs which are covalently linked by a peptide bond or an analog of a peptide bond. The term peptide includes oligomers and polymers of amino acids or amino acid analogs. The term peptide also includes molecules which are commonly referred to as peptides, which generally contain from about two (2) to about twenty (20) amino acids. The term peptide also includes molecules which are commonly referred to as polypeptides, which generally contain from about twenty (20) to about fifty amino acids (50). The term peptide also includes molecules which are commonly referred to as proteins, which generally contain from about fifty (50) to about three thousand (3000) amino acids. The amino acids of the peptide may be L-amino acids or D-amino acids. A peptide, polypeptide or protein may be synthetic, recombinant or naturally occurring. A synthetic peptide is a peptide which is produced by artificial means *in vitro*.

The terms "oligosaccharide" and "OS" antigen refer to a carbohydrate comprising up to ten component sugars, either O or N linked to the next sugar. Likewise, the terms "polysaccharide" and "PS" antigen refer to polymers of more than ten monosaccharide residues linked glycosidically in branched or unbranched chains.

5 The terms "microbial sequence" and "sequence of a microbe" refers to synthetic, recombinant or purified sequences (preferably sequence fragments comprising at least one B cell epitope) of a virus, a bacterium, a fungus, and a parasite. Exemplary microbial sequences include those of Influenza A, *Staphylococcus sp.*, *Candida sp.*, and *Plasmodium sp.*.

As used herein, the term "mammalian sequence" refers to synthetic, recombinant or  
10 purified sequences (preferably sequence fragments comprising at least one B cell epitope) of a mammal. Exemplary mammalian sequences include cytokine sequence, MHC class I heavy chain sequences, MHC class II alpha and beta chain sequences, and amyloid  $\beta$ -peptide sequences.

15 The term "allergen" as used herein, refers to an antigenic substance capable of producing an immediate type hypersensitivity reaction (allergy) in a subject. Exemplary allergens include food allergens such as peanut allergen, grass pollen allergen and dust mite allergen.

The term "particle" as used herein refers to a virus-like protein structure of approximately 25-35 nm in diameter, into which hepadnavirus core polypeptides spontaneously assemble.  
20 Particle formation is measured by the exemplary methods for assessing hepadnavirus core antigen expression and assembly disclosed herein.

The term "aggregate" as used herein refers to a cluster, clump, or mass of individual polypeptides and/or particles.

As used herein, the terms "immune enhancer" and "molecular adjuvant" refer to molecules which provide a co-stimulus to B cells or other antigen presenting cells, thereby  
25 increasing the level of the immune response by the cells to an antigen. Exemplary immune enhancers include but are not limited to unmethylated CpG dinucleotides, CD40 ligand, complement C3d fragment, BAFF, and LAG-3.

The terms "mammals" and "mammalian" refer animals of the class mammalia which nourish their young by fluid secreted from mammary glands of the mother, including human  
30 beings. The class "mammalian" includes placental animals, marsupial animals, and monotrematal animals. An exemplary "mammal" may be a rodent, primate (including simian

and human) ovine, bovine, ruminant, lagomorph, porcine, caprine, equine, canine, feline, ave, etc. Preferred non-human animals are selected from the order Rodentia.

Preferred embodiments of the present invention are primarily directed to vertebrate (backbone or notochord) members of the animal kingdom.

5 The terms "patient" and "subject" refer to a mammal that may be treated using the methods of the present invention.

The term "control" refers to subjects or samples which provide a basis for comparison for experimental subjects or samples. For instance, the use of control subjects or samples permits determinations to be made regarding the efficacy of experimental procedures. In some 10 embodiments, the term "control subject" refers to a subject that which receives a mock treatment (e.g., saline alone or WHcAg without a heterologous antigen insert or conjugate).

As used herein, the term "immune response" refers to the reactivity of an organism's immune system in response to an antigen. In vertebrates, this may involve antibody production, induction of cell-mediated immunity, and/or complement activation (e.g., phenomena associated 15 with the vertebrate immune system's prevention and resolution of infection by microorganisms). In preferred embodiments, the term immune response encompasses but is not limited to one or more of a "lymphocyte proliferative response," a "cytokine response," and an "antibody response."

In particularly preferred embodiments, the immune response is largely reactive with an 20 antigen of interest. For instance, when used in reference to administration of a hybrid WHcAg-NANP vaccine to a mammalian subject, the term refers to the immune response produced in the subject which reacts with either the WHcAg core or the NANP insert/conjugate of the vaccine. Immune responses reactive with an antigen of interest are measured *in vitro* using various methods disclosed herein.

25 The term "reactive with an antigen of interest" when made in reference to an immune response refers to an increased level of the immune response to the antigen of interest as compared to the level of the immune response to control antigen. (e.g., unrelated antigen).

The term "lymphocyte proliferative response" refers to antigen-induced lymphocyte (e.g., PBL) increase in cell number. Alternatively, or in addition, the term "proliferation" refers to the 30 physiological and morphological progression of changes that cells undergo when dividing, for instance including DNA replication as measured by tritiated thymidine incorporation.

The term "cytokine response" refers to antigen-induced cytokine secretion by lymphocytes as measured for instance by assaying culture supernatants for cytokine content (e.g., IL-2, IFN $\gamma$ , TNF $\alpha$ , IL-4, etc) by ELISA.

The term "antibody response" refers to the production of antibodies (e.g., IgM, IgA, IgG)  
5 which bind to an antigen of interest, this response is measured for instance by assaying sera by antigen ELISA.

The term "adjuvant" as used herein refers to any compound which, when injected together with an antigen, non-specifically enhances the immune response to that antigen.

Exemplary adjuvants include but are not limited to incomplete Freunds adjuvant (IFA),  
10 aluminum-based adjuvants (e.g., AlOH, AlPO<sub>4</sub>, etc), and Montanide ISA 720.

The terms "diluent" and "diluting agent" as used herein refer to agents used to diminish the strength of an admixture. Exemplary diluents include water, physiological saline solution, human serum albumin, oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents, antibacterial agents such as benzyl alcohol, antioxidants such as ascorbic acid or sodium bisulphite, chelating agents such as ethylene diamine-tetra-acetic acid, buffers such as acetates, citrates or phosphates and agents for adjusting the osmolarity, such as sodium chloride or dextrose.  
15

The terms "carrier" and "vehicle" as used herein refer to usually inactive accessory substances into which a pharmaceutical substance (e.g., WHcAg vaccine) is suspended.  
20 Exemplary carriers include liquid carriers (such as water, saline, culture medium, saline, aqueous dextrose, and glycols) and solid carriers (such as carbohydrates exemplified by starch, glucose, lactose, sucrose, and dextrans, anti-oxidants exemplified by ascorbic acid and glutathione, and hydrolyzed proteins).

The term "derived" when in reference to a peptide derived from a source (such as a  
25 microbe, cell, etc.) as used herein is intended to refer to a peptide which has been obtained (e.g., isolated, purified, etc.) from the source. Alternatively, or in addition, the peptide may be genetically engineered and/or chemically synthesized.

The terms "operably linked," "in operable combination," and "in operable order" as used herein refer to the linkage of nucleic acid sequences such that they perform their intended  
30 function. For example, operably linking a promoter sequence to a nucleotide sequence of interest refers to linking the promoter sequence and the nucleotide sequence of interest in a

manner such that the promoter sequence is capable of directing the transcription of the nucleotide sequence of interest and/or the synthesis of a polypeptide encoded by the nucleotide sequence of interest. Similarly, operably linking a nucleic acid sequence encoding a protein of interest means linking the nucleic acid sequence to regulatory and other sequences in a manner such that the 5 protein of interest is expressed. The term also refers to the linkage of amino acid sequences in such a manner so that a functional protein is produced.

The terms "C-terminal portion," "COOH-terminal portion," "carboxy terminal portion," "C-terminal domain," "COOH-terminal domain," and "carboxy terminal domain," when used in reference to an amino acid sequence of interest (such as WHcAg) refer to the amino acid 10 sequence (and portions thereof) that is located from approximately the middle of the amino acid sequence of interest to the C-terminal-most amino acid residue of the sequence of interest. For example, the C-terminal portion of WHcAg includes the amino acid sequence from position 150 to 188 of WHcAg (SEQ ID NO:2); the C-terminal portion of GSHcAg includes the amino acid sequence from position 149 to 187 of GSHcAg (SEQ ID NO:22); the C-terminal portion of 15 HBCAg includes the amino acid sequence from position 150 to 183 of HBcAg (SEQ ID NO:42).

The terms "specific binding," "binding specificity," and grammatical equivalents thereof when made in reference to the binding of a first molecule (such as a polypeptide, glycoprotein, nucleic acid sequence, etc.) to a second molecule (such as a polypeptide, glycoprotein, nucleic acid sequence, etc.) refer to the preferential interaction between the first molecule with the 20 second molecule as compared to the interaction between the second molecule with a third molecule. Specific binding is a relative term that does not require absolute specificity of binding; in other words, the term "specific binding" does not require that the second molecule interact with the first molecule in the absence of an interaction between the second molecule and the third molecule. Rather, it is sufficient that the level of interaction between the first molecule 25 and the second molecule is higher than the level of interaction between the second molecule with the third molecule. "Specific binding" of a first molecule with a second molecule also means that the interaction between the first molecule and the second molecule is dependent upon the presence of a particular structure on or within the first molecule; in other words the second molecule is recognizing and binding to a specific structure on or within the first molecule rather 30 than to nucleic acids or to molecules in general. For example, if a second molecule is specific for structure "A" that is on or within a first molecule, the presence of a third nucleic acid

sequence containing structure A will reduce the amount of the second molecule which is bound to the first molecule.

As used in this specification and the appended claims, the singular forms "a," "an" and "the" includes both singular and plural references unless the content clearly dictates otherwise.

5 For example, the term "inserted at a position" as used herein in reference to a polypeptide sequence refers to insertion at one or more (such as one, two, three, etc.) amino acid positions in the polypeptide sequence. In one preferred embodiment, insertion is at one amino acid position as exemplified herein.

The phrase "chosen from A, B, and C" as used herein, means selecting one or more of A,  
10 B, C.

As used herein, the term "or" when used in the expression "A or B," where A and B refer to a composition, disease, product, *etc.*, means one, or the other, or both.

The term "on" when in reference to the location of a first article with respect to a second article means that the first article is on top and/or into the second article, including, for example, 15 where the first article permeates into the second article after initially being placed on it.

As used herein, the term "comprising" when placed before the recitation of steps in a method means that the method encompasses one or more steps that are additional to those expressly recited, and that the additional one or more steps may be performed before, between, and/or after the recited steps. For example, a method comprising steps a, b, and c encompasses a  
20 method of steps a, b, x, and c, a method of steps a, b, c, and x, as well as a method of steps x, a, b, and c. Furthermore, the term "comprising" when placed before the recitation of steps in a method does not (although it may) require sequential performance of the listed steps, unless the content clearly dictates otherwise. For example, a method comprising steps a, b, and c encompasses, for example, a method of performing steps in the order of steps a, c, and b, the  
25 order of steps c, b, and a, and the order of steps c, a, and b, *etc.*

Unless otherwise indicated, all numbers expressing quantities of ingredients, properties such as molecular weight, reaction conditions, and so forth as used in the specification and claims are to be understood as being modified in all instances by the term "about." Accordingly, unless indicated to the contrary, the numerical parameters in the specification and claims are  
30 approximations that may vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and without limiting the application of the doctrine of

equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques. Notwithstanding that the numerical ranges and parameters describing the broad scope of the invention are approximation, the numerical values in the specific examples are reported as precisely as possible. Any numerical value, however, inherently contains standard deviations that necessarily result from the errors found in the numerical value's testing measurements.

The term "not" when preceding, and made in reference to, any particularly named molecule (mRNA, *etc.*) or phenomenon (such as biological activity, biochemical activity, *etc.*) means that only the particularly named molecule or phenomenon is excluded.

10 The term "altering" and grammatical equivalents as used herein in reference to the level of any substance and/or phenomenon refers to an increase and/or decrease in the quantity of the substance and/or phenomenon, regardless of whether the quantity is determined objectively, and/or subjectively.

15 The term "increase," "elevate," "raise," and grammatical equivalents when in reference to the level of a substance and/or phenomenon in a first sample relative to a second sample, mean that the quantity of the substance and/or phenomenon in the first sample is higher than in the second sample by any amount that is statistically significant using any art-accepted statistical method of analysis. In one embodiment, the increase may be determined subjectively, for example when a patient refers to their subjective perception of disease symptoms, such as pain, 20 clarity of vision, *etc.*. In another embodiment, the quantity of the substance and/or phenomenon in the first sample is at least 10% greater than the quantity of the same substance and/or phenomenon in a second sample. In another embodiment, the quantity of the substance and/or phenomenon in the first sample is at least 25% greater than the quantity of the same substance and/or phenomenon in a second sample. In yet another embodiment, the quantity of the 25 substance and/or phenomenon in the first sample is at least 50% greater than the quantity of the same substance and/or phenomenon in a second sample. In a further embodiment, the quantity of the substance and/or phenomenon in the first sample is at least 75% greater than the quantity of the same substance and/or phenomenon in a second sample. In yet another embodiment, the quantity of the substance and/or phenomenon in the first sample is at least 90% greater than the 30 quantity of the same substance and/or phenomenon in a second sample.

The terms "reduce," "inhibit," "diminish," "suppress," "decrease," and grammatical equivalents when in reference to the level of a substance and/or phenomenon in a first sample relative to a second sample, mean that the quantity of substance and/or phenomenon in the first sample is lower than in the second sample by any amount that is statistically significant using 5 any art-accepted statistical method of analysis. In one embodiment, the reduction may be determined subjectively, for example when a patient refers to their subjective perception of disease symptoms, such as pain, clarity of vision, etc.. In another embodiment, the quantity of substance and/or phenomenon in the first sample is at least 10% lower than the quantity of the same substance and/or phenomenon in a second sample. In another embodiment, the quantity of 10 the substance and/or phenomenon in the first sample is at least 25% lower than the quantity of the same substance and/or phenomenon in a second sample. In yet another embodiment, the quantity of the substance and/or phenomenon in the first sample is at least 50% lower than the quantity of the same substance and/or phenomenon in a second sample. In a further embodiment, the quantity of the substance and/or phenomenon in the first sample is at least 75% 15 lower than the quantity of the same substance and/or phenomenon in a second sample. In yet another embodiment, the quantity of the substance and/or phenomenon in the first sample is at least 90% lower than the quantity of the same substance and/or phenomenon in a second sample.

Reference herein to any specifically named protein (such as "WHcAg," "GSHcAg," and "HBcAg," etc.) refers to a polypeptide having at least one of the biological activities of the 20 specifically named protein, wherein the biological activity is detectable by any method. In addition, reference herein to any specifically named protein (such as "WHcAg," "GSHcAg," and "HBcAg," etc.) includes within its scope fragments, fusion proteins, and variants of the specifically named protein. The term "variant" of a protein as used herein is defined as an amino acid sequence which differs by insertion, deletion, and/or conservative substitution of one or 25 more amino acids from the protein. In one embodiment, the sequence of the variant has at least 99% identity, preferably at least 95% identity, and more preferably at least 90% identity with the sequence of the protein in issue.

For example, the term "has the biological activity of a specifically named protein" (such as "WHcAg," "GSHcAg," and "HBcAg," etc.) when made in reference to the biological activity 30 of a variant of the specifically named protein refers, for example, to a quantity of binding of an antibody that is specific for the specifically named protein to the variant which is preferably

greater than 50% (preferably from 50% to 500%, more preferably from 50% to 200%, most preferably from 50% to 100%), as compared to the quantity of binding of the same antibody to the specifically named protein.

Reference herein to any specifically named nucleotide sequence (such as a sequence 5 encoding WHcAg, a sequence encoding GSHcAg, and a sequence encoding HBcAg, etc.) includes within its scope fragments, homologs, and sequences that hybridize under stringent condition to the specifically named nucleotide sequence. The term "homolog" of a specifically named nucleotide sequence refers to an oligonucleotide sequence which exhibits greater than or equal to 50% identity to the sequence of interest. Alternatively, or in addition, a homolog of any 10 specifically named nucleotide sequence (such as a sequence encoding WHcAg, a sequence encoding GSHcAg, and a sequence encoding HBcAg, etc.) is defined as an oligonucleotide sequence which has at least 95% identity with the sequence of the nucleotide sequence in issue. In another embodiment, the sequence of the homolog has at least 90% identity, and preferably at least 85% identity with the sequence of the nucleotide sequence in issue.

15

#### **DETAILED DESCRIPTION OF THE INVENTION**

The present invention relates to rodent hepatitis virus core proteins and nucleic acids. In particular, the present invention provides compositions and methods comprising recombinant rodent hepatitis virus core proteins or nucleic acids for use in vaccine formulations.

20

##### **I. Hepatitis B Virus Core Antigen (HBcAg)**

The human hepatitis B virus core antigen is a 21 kDa polypeptide. Two kinds of HBcAg core particles are spontaneously assembled during the course of virion assembly and during heterologous expression in both prokaryotic and eukaryotic systems: particles with T = 4 25 symmetry containing 240 polypeptides, and particles with T = 3 symmetry containing 180 polypeptides. Recent cryoelectron microscopy studies have revealed the structure of HBcAg particles to a resolution of 7.4 angstroms (Botcher *et al.*, Nature, 386:88-91, 1997; and Conway *et al.*, Nature, 386:91-94, 1997). Dimer clustering of HBcAg subunits produces spikes on the surface of the core shell, which consist of radial bundles of four long  $\alpha$ -helices. The 30 immunodominant B cell epitope on HBcAg is localized around amino acids 76-82 (Salfeld *et al.*, J Virol, 63:798-808, 1989; and Schodel *et al.*, J Virol, 66:106-114, 1992), apparently forming a

loop connecting adjacent helices (See, Figure 1). The spacing of the spikes on the core shell is optimal for B cell mIg receptor cross-linking. In addition, the inherent immunogenicity of the native HBcAg B cell epitopes suggested the desirability of substituting heterologous epitopes in the same position (*i.e.*, at the tip of the spike). In fact, a number of pathogen-specific B cell epitopes have been chemically linked or fused by recombinant methods to HBcAg in order to increase their immunogenicity (See, Milich *et al.*, Ann NY Acad Sci, 754:187-201, 1995; and Pumpens *et al.*, Intervirology, 38:63-74, 1995, for reviews). These studies, conducted by a number of independent laboratories, have met with significant success including complete protection against foot and mouth disease virus (Clarke *et al.*, Nature, 330:381-384, 1987),  
5 *Plasmodium berghei* (Schodel *et al.*, J Exp Med, 180:1037-1046, 1994), and *Plasmodium yoelii* (Schodel *et al.*, Behring Inst Mitt, 114-119, 1997).

10 Adjuvants are broadly separated into two classes based upon their primary mechanism of action: vaccine delivery systems (*e.g.* emulsions, microparticles, iscoms, liposomes, etc.) that target associated antigens to antigen presenting cells (APC); and immunostimulatory adjuvants (e.g., LPS, MLP, CpG, etc.) that directly activate innate immune responses. The HBcAg platform provides a delivery system that targets antigen-specific B cells and other primary APC,  
15 as well as efficient T cell help for antigen-specific B cells. Additionally, the core platform functions as an immunostimulatory adjuvant by directly activating antigen-specific B cells by virtue of cross-linking membrane immunoglobulin (mIg) receptors for induction of B7.1 and  
20 B7.2 costimulatory molecule expression on naive resting B cells (Milich *et al.*, Proc Natl Acad Sci USA, 94:14648-14653, 1997).

## II. Rodent Hepatitis Virus Core Platform

### 25 A. Woodchuck Hepatitis Virus (WHV)

During development of the present invention, comparative studies between the HBcAg and the WHcAg were undertaken. The WHcAg is only 67% identical at the amino acid level to the HBcAg, and the comparative studies revealed that, the HBcAg and WHcAg proteins do not significantly crossreact at the antibody level and only partially at the CD4<sup>+</sup> T cell level.  
30 However, these two particulate antigens share a number of characteristics including: enhanced immunogenicity of T cell-independent, as well as T cell-dependent antibody production; the

absence of nonresponder MHC-haplotypes; efficient antigen-specific B cell activation; the ability of naive WHcAg or HBcAg-specific B cells to act as primary APC for naive core-specific Th cells; and the ability to act as a carrier moiety for foreign epitopes. In fact, in a direct comparison of an HBcAg-CS vaccine candidate (V12.PF3.1) and a WHcAg-based hybrid

5 particle containing the same CS repeat sequence (188-M74), a single 20 µg dose of the WHcAg hybrid particle in IFA elicited significantly higher levels of anti-NANP antibodies with a better persistence profile than the same dose of the HBcAg hybrid vaccine (See, Figure 2). Furthermore, the WHcAg appears to tolerate insertions of foreign epitopes at a greater number of positions than the HBcAg, as illustrated in Figure 3. A number of internal insertions inside the

10 loop region (positions 76-82), as well as internal insertions outside the loop region were tolerated by WHcAg. This is in sharp contrast to the rather limited number of efficient insertion sites described for the HBcAg, including those in loop positions 77, 78, 81, 82 (Pumpens and Grens, Intervirology, 44:98-114, 2001). Importantly, the identification of an expanded number of insertion sites was dependent on additional modifications to the C-terminus that stabilize the

15 internal insertions. Indeed, 21 separate C-terminal modifications (See, Table 1) have been generated for use in combination with 17 insertion sites, to ensure efficient hybrid WHcAg particle assembly. Additionally, the insert sequence was found to effect hybrid WHcAg assembly competence. For example, highly positively-charged epitope inserts tended to destabilize hybrid particle assembly. Thus, three variables relevant to the design of hybrid

20 hepadnavirus core particles have been identified including insert position, C-terminus and epitope sequence.

**Table 1. Sequences of the C-Termini of the Woodchuck Vaccine Platform<sup>1</sup>**

Designation	WHcAg C-Terminal Sequence	Identifier
wild type	RRGGARASRSPRRTPSPRRRSQSPRRRSQSPSANC	SEQ ID NO:2
150R	R	N/A
150C	C	N/A
150-2RC	RRC	N/A
150-3RC	RRRC	SEQ ID NO:3
150-4RC	RRRRC	SEQ ID NO:4
150-3KC	KKKC	SEQ ID NO:5
150-3AC	AAAC	SEQ ID NO:6
WT-R	AAGGARASRSPSQSPSQSPSANC	SEQ ID NO:7
WT-R1	AAGGARASRSQSPSQSPSANC	SEQ ID NO:8
WT-R2	AAGGARASRSQSSQSPSANC	SEQ ID NO:9
WT-R3	AAGGARASRSQSSQSSANC	SEQ ID NO:10
C-Long	RRGGARASQSPSANC	SEQ ID NO:11
C-Long(M1)	ARGGARASQSPSANC	SEQ ID NO:12
C-Long(M2)	RAGGARASQSPSANC	SEQ ID NO:13
C-Long(M3)	AAGGARASQSPSANC	SEQ ID NO:14
HyW	AAGRSPSQSPSQRESQC	SEQ ID NO:15
HyW-1	AAGRSPSQSPSQSPSANC	SEQ ID NO:16
HyW-2	AAGRSPSQSPSQSSANC	SEQ ID NO:17
HyW-3	AAGRSQSPSQSSANC	SEQ ID NO:18
HyW-4	AAGRSPSQSSQSSANC	SEQ ID NO:19
HyW-5	AAGRSQSSQSSANC	SEQ ID NO:20

<sup>1</sup>The wild type C-terminal protein sequence corresponds to positions 150-188. The full length protein sequence of WHcAg is set forth herein as SEQ ID NO:1, while the full length DNA sequence is set forth as SEQ ID NO:37. Additionally, the wild type N-terminal protein sequence (corresponding to positions 1-149) is set forth as SEQ ID NO:38.

A combinatorial approach was made feasible by development of an ELISA-based screening system to detect core protein expression level, insert antigenicity and particle assembly in the lysates of transformed bacteria, prior to purification. Although a *Plasmodium* circumsporozoite (CS) repeat was used as a model epitope, this technology is not confined to a limited set of epitopes. In fact, insertion of 22 out of 24 different epitopes into the WHcAg platform has been successfully accomplished during development of the present invention. Another bottleneck that had existed in the characterization of hybrid core particles was the necessity for *in vivo* immunogenicity testing requiring 4-6 weeks for the analysis of a primary response. This bottleneck has been widened during development of the present invention by utilizing *in vitro* antibody production as a correlate of *in vivo* immunogenicity. The *in vitro* antibody production assay requires just 5 days of tissue culture. Establishment of *in vitro* antibody production as a predictor of *in vivo* immunogenicity is a powerful screening tool dramatically shortening the time necessary for the development of hybrid WHcAg particle vaccine candidates. Subsequently, once a vaccine candidate is shown to induce antibody production *in vitro*, then *in vivo* studies of dose, route and formulation are completed.

Three categories of model antigens are contemplated to be successfully accommodated by the WHcAg platform system including: i) peptidic epitopes inserted into WHcAg by recombinant methods; ii) polysaccharide (PS) antigens chemically conjugated to directly to or lysine-modified WHcAg particles; and iii) larger, non-linear protein/polypeptide antigens incorporated into WHcAg by recombinant or chemical methods. In some embodiments, the incorporation of larger protein sequences is accomplished by production of mosaic WHcAg particles comprised of an optimal mixture of wild-type WHcAg and WHcAg-fusion proteins containing the desired inserted sequence. This mosaic approach is also suitable for utilization of so-called molecular adjuvants through linkage to the C-terminal amino acid residue of WHcAg particles. A number of useful molecular adjuvants, which bridge the gap between innate and adaptive immunity, have in common the ability to provide a co-stimulus targeting immune cells (typically B cells or other APCs). Linkage of a molecular adjuvant to a hybrid WHcAg particle is contemplated to be advantageous in that only the antigen-specific B cell or APC taking up the particle become activated, as opposed to the non-specific activation induced by merely mixing adjuvant and antigen.

Additionally in other embodiments, the hepadna virus core platforms are utilized in non-infectious disease situations, such as those requiring high level *in vivo* antibody production (as an alternative to monoclonal antibody therapy). For example, active immunization to elicit anti-TNF $\alpha$  therapeutic autoantibodies is contemplated to have a number of advantages over 5 monoclonal anti-TNF $\alpha$  therapy for the treatment of arthritis and other inflammatory diseases.

Without limiting the invention, advantages of using modified WHcAg particle vaccine provided by the present invention include: i) WHcAg is equally or more immunogenic than the HBcAg at the T and B cell levels; ii) WHcAg will not substantially compromise the use of the anti-HBc diagnostic assay because the WHcAg and HBcAg are not substantially crossreactive at 10 the antibody level; iii) immune tolerance in HBV chronic carriers can be circumvented by the use of the WHcAg platform because the HBcAg and WHcAg are only partially crossreactive at the T cell level; and iv) the WHcAg combinatorial technology is more versatile than the HBcAg in terms of accommodating the insertion of a greater variety of foreign epitopes (See, Table 2).

**Table 2. Summary of Advantages of Using WHcAg as a Vaccine Platform**

No.	Advantage
1	Efficient self-assembly into 25-35 nm particles allowing for multivalency of inserted epitopes and combination vaccines.
2	Highly immunogenic during natural infection and vaccination.
3	Only 1-2 doses required in animal models.
4	A library comprising 17 insertion positions and 21 C-terminal modifications is provided by the current invention.
5	A combinatorial technology involving insert position, C-terminus and foreign sequence is provided by the current invention.
6	Linker residues permitting assembly of core particles containing destabilizing foreign sequences is provided by the current invention.
7	Th cell as well as B cell epitopes are accommodated on hybrid particles.
8	Hybrid particles elicit a broad spectrum of IgG isotypes.
9	Hybrid particles do not require an adjuvant, although immunogenicity can be enhanced by a metabolizable oil/alum depot effect.
10	Core particles can accommodate incorporation of a molecular adjuvant.
11	Core particles can accommodate linkage of carbohydrate antigens and large non-linear protein antigens.
12	Hybrid particles are very stable (e.g., a cold chain is not necessarily required).
13	Use of core particles does not compromise the anti-HBc diagnostic assay.
14	Use of core particles avoids the problem of immune tolerance in HBV-infected individuals.
15	Recombinant core particles can be produced in <i>E. coli</i> , which is cost effective and scaleable.

### **B. Ground Squirrel Hepatitis Virus (GHV)**

In another embodiment, a second new combinatorial platform technology is developed by modification of the ground squirrel hepadna virus (GHV) core protein (GSHcAg). The GSHcAg is 91% identical at the amino acid level to the WHcAg. Modifications to the C-terminus of the  
5 of the GSHcAg protein, similar to those described above for WHcAg, are made as shown in Table 3.

**Table 3. Sequences of the C-Termini of the Ground Squirrel Vaccine Platform<sup>1</sup>**

Designation	GSHcAg C-Terminal Sequence	Identifier
wild type	RRRGGSRAARSPRRRTSPSPRRRSQSPRRRSQSPASNC	SEQ ID NO:22
150R	R	N/A
150C	C	N/A
150-2RC	RRC	N/A
150-3RC	RRRC	SEQ ID NO:3
150-4RC	RRRC	SEQ ID NO:4
150-3KC	KKKC	SEQ ID NO:5
150-3AC	AAAC	SEQ ID NO:6
WT-R	AAGGSRAARSPSQSPSQSPASNC	SEQ ID NO:23
WT-R1	AAGGSRAARSQSPSQSPASNC	SEQ ID NO:24
WT-R2	AAGGSRAARSQSSQSPASNC	SEQ ID NO:25
WT-R3	AAGGSRAARSQSSQSASNC	SEQ ID NO:26
C-Long	RRGGSRAAQSPASNC	SEQ ID NO:27
C-Long(M1)	ARGGSRASQSPASNC	SEQ ID NO:28
C-Long(M2)	RAGGSRASQSPASNC	SEQ ID NO:29
C-Long(M3)	AAGGSRASQSPASNC	SEQ ID NO:30
HyW	AAGRSPSQSPSQRESQC	SEQ ID NO:31
HyW-1	AAGRSPSQSPSQSPASNC	SEQ ID NO:32
HyW-2	AAGRSPSQSPSQSASNC	SEQ ID NO:33
HyW-3	AAGRSPSQSPSQSASNC	SEQ ID NO:34
HyW-4	AAGRSPSQSSQSASNC	SEQ ID NO:35
HyW-5	AAGRSPSQSSQSASNC	SEQ ID NO:36

<sup>1</sup> The wild type C-terminal sequence corresponds to positions 149-187. The full length protein sequence of GSHcAg is set forth herein as SEQ ID NO:21, while the full length DNA sequence is set forth as SEQ ID NO:39. Additionally, the wild type N-terminal protein sequence (corresponding to positions 1-148) is set forth as SEQ ID NO:40.

### III. Human Hepatitis B Virus (HBV) Core Platform

In a further embodiment, the human hepatitis B virus (HBV) core antigen (HBcAg) platform is improved through introduction of various modifications. Modifications to the C-terminus of the HBcAg protein, are made as shown in Table 4.

5

**Table 4. Sequences of the C-Termini of the Human HBcAg Vaccine Platform**

Designation	HBcAg C-Terminal Sequence	Identifier
wild type	RRRGRSPRRRTSPSPRRRSQSPRRRSQSRESQC	SEQ ID NO:42
150R	R	N/A
150C	C	N/A
150-2RC	RRC	N/A
150-3RC	RRRC	SEQ ID NO:3
150-4RC	RRRRC	SEQ ID NO:4
150-3KC	KKC	SEQ ID NO:5
150-3AC	AAAC	SEQ ID NO:6
WT-R	AAGRSPSQSPSQSRESQC	SEQ ID NO:43
WT-R1	AAGRSQSPSQSRESQC	SEQ ID NO:44
WT-R2	AAGRSQSSQSRESQC	SEQ ID NO:45
WT-R3	AAGRSQSSQSESQC	SEQ ID NO:46
C-Long	RRGSQSRESQC	SEQ ID NO:47
C-Long(M1)	ARGSQSRESQC	SEQ ID NO:48
C-Long(M2)	RAGSQSRESQC	SEQ ID NO:49
C-Long(M3)	AAGSQSRESQC	SEQ ID NO:50
HyW	AAGRSPSQSPSQSPSANC	SEQ ID NO:51
HyW-1	AAGRSPSQSPSQSRESQC	SEQ ID NO:52
HyW-2	AAGRSPSQSPSQSESQC	SEQ ID NO:53
HyW-3	AAGRSQSPSQSESQC	SEQ ID NO:54
HyW-4	AAGRSPSQSSQSESQC	SEQ ID NO:55
HyW-5	AAGRSQSSQSESQC	SEQ ID NO:56

<sup>1</sup> The wild type C-terminal sequence corresponds to positions 150-183. The full length protein sequence of HBcAg is set forth herein as SEQ ID NO:41, while the full length DNA sequence is set forth as SEQ ID NO:57. Additionally, the wild type N-terminal protein sequence (corresponding to positions 1-149) is set forth as SEQ ID NO:58.

5

#### IV. Additional Modifications to the WHcAg Vaccine Platform

In some embodiments, the 188 amino acid wild-type (WT) WHcAg is further modified by recombinant technology to increase the number of potential insertion sites. The WT WHcAg core gene is modified by creating and inserting unique cloning restriction sites at positions of interest along its sequence. Insert sites are produced to accept any nucleic acid sequences flanked by the conserved ends. The number of sites per core gene is varied to obtain bi or multivalent particles. In particular additional insertion sites are created in the following locations: i) in the alpha-helical core regions forming the stem of the spike, and ii) in the N-terminal and non-helical regions of the protein. Different cloning restriction sites are used at different positions, and different linkers are used with different heterologous inserted sequences.

The pUCWHc vector expressing the WHcAg sequence under the control of the Lac Operon promoter is inserted between *NcoI-BamH1* sites for subcloning convenience. The foreign inserted sequences are designed as follows: i) for small linear peptidic epitopes, direct synthesis of the corresponding nucleotide sequences is done in order to flank the foreign sequence with the desired unique restriction site(s) created on the plasmid; ii) for larger protein fragments, the foreign sequence is first obtained by PCR from plasmids encoding the complete corresponding gene, and modified accordingly with unique flanking restriction sites.

##### 25 A. Creation of New Insertion Sites

All insertions are accomplished by either using the *EcoR1-Xho1* sites or *SacI* sites, with only the position of the insertion differing between constructs (See, Figure 20). Accordingly, new primers/oligonucleotides are designed in order to encompass either one or two restriction sites present on the wild-type WHcAg gene and to code for 5'*EcoR1*-3'*Xho1* sites or *SacI* sites.

30

### **1. Direct Synthesis of Sense and Antisense Oligonucleotides**

The oligonucleotides span two other WHcAg gene restriction sites (e.g., RS1, RS2) and do not exceed 100 nucleotides in length (limit for direct synthesis). Both the plasmid pUC-WT and the oligonucleotides are then digested by the RS1 and RS2 enzymes and purified from a low-melting point agarose gel. The RS1-*EcoR1-Xho1*-RS2 fragment replaces the corresponding RS1-RS2 sequence of the pUC-WT plasmid via ligation to produce pUC-WT $\Delta$  insertion site (e.g., pUC WT $\Delta$  98-99 corresponds to WT WHcAg gene with insertion between amino acids 98 and 99).

### **10 2. Design of PCR Primers**

In some instances, the direct synthesis of oligonucleotides cannot be realized because the desired insertion site is distanced from one of two usable restriction sites of the WHcAg gene by more than 100 nucleotides. In these cases, one of the primers (forward or reverse depending on the desired orientation) is designed to match the exact sequence of the WT WHcAg gene and to include a unique restriction site (RS3). The other primer is designed to create a mismatch (PCR mutagenesis) from the WT sequence and to introduce *EcoR1-Xho1* sites, plus an RS4 site belonging to the WT gene only. PCR with the forward-RS3, and the reverse-*EcoR1-Xho1*-RS4 primers is performed using the pUC-WT plasmid as a template. The resulting PCR product and the pUC-WT plasmid are then digested by RS3-RS4 and ligated to create the new pUC-WT $\Delta$  insertion site. Linkers are used when necessary to accommodate foreign sequences such as for insertion of large fragments (Kratz *et al.*, Proc Natl Acad Sci USA, 96:1915-1920, 1999). By creating 5' *EcoR1-Xho1* 3' as insertion sites, and keeping the same reading frame as that of the WT WHcAg gene, each foreign sequence is flanked by the same linker, Gly-Ile-Leu on their N-terminus, and Leu-Glu on their C-terminus. Similarly by creating 5' SacI-SacI 3' as insertion sites, and keeping the same reading frame as that of the WT WHcAg gene, each foreign sequence is flanked by the same linker, Ser-Ser, on both their N- and C- termini. The following primer sequence containing both *EcoR1* and *Xho1* restriction sites, GGAAATTCTTCTCCTCGAG (SEQ ID NO:63) is used for this purpose. Similarly, others sequences are introduced to code for new linkers (e.g., Gly4-S-Gly4) on each side of the foreign sequence.

## B. Modifications of the C-Terminus

The library of C-termini is expanded to eliminate certain motifs (e.g., RNA/DNA binding motifs) and to accommodate the addition of other linker/spacer sequences. As described below in the examples, modifications of the C-terminus that enhance expression/assembly and/or 5 antigenicity/immunogenicity of various hybrid core constructs have been characterized. The new C-termini are modified by designing oligonucleotides encoding the sequence of interest and flanked by 5' *Bse*A1 and 3' *Bam*H1 sites as a general pattern, and then using the oligonucleotides to replace the corresponding native fragment on the pUC-WT plasmid. All the WHcAg constructs (insert sites, C-termini, +/- foreign sequences) are sequenced in both 10 directions at an automated sequencing facility. The hybrid WHcAg constructs (pUC vectors) are then used to transform chemically-competent Top10 *E. coli* by heat shock. The transformed Top10 grow overnight at low temperature 28°C to avoid inclusion body formation, before the expression of the protein is induced by addition of IPTG (1mM for 4h). The bacteria are lysed in a lysozyme-salt solution containing proteolysis inhibitors. The resulting supernatant is 15 precipitated overnight in the cold with 50% ammonium sulfate. The proteins are then purified by chromatography on hydroxylapatite and Sepharose 4B columns. In some embodiments, for better and tighter control of the expression, each hybrid WHcAg construct is subcloned into another expression vector, pET11d, at the *Ncol-Bam*H1 sites. The pET11d vector allows expression of the corresponding protein under an inducible T7/Lac Operon promoter. These 20 hybrid constructs are then transformed in the BL21 (DE3) *E. coli* strain.

## C. Rapid Screening Technology

The approach of combining the optimal C-terminus from a selection of 21 termini and the optimal insert position from a choice of 17 positions, in the context of a given epitope requires a 25 rapid screening technology that can be applied early in the manufacturing process. Therefore, an antibody-based method for detecting expression of the core polypeptide, assembly of the polypeptide into core hybrid particles and for assessing antigenicity of the inserted heterologous epitope has been developed. This rapid screening technique is applied to lysates of the transformed *E. coli* to assess the desirability of any given hybrid core before a significant 30 investment in vaccine production is made. As described in the examples, capture ELISAs were designed either to detect the WHcAg polypeptide as a marker of expression or to detect the

WHcAg particle as a marker for assembly, while insert-specific mAbs were used to assess the expression level and antigenicity of the insert (*See, Figure 21*). Lysates were sequentially screened with mAbs that preferentially recognize denatured WHcAg (anti-p WHc), assembled WHcAg particles (anti-nWHc), and proper display of insert sequence (insert-specific mAbs).

5 Based on relative assembly scores of the lysates, optimal hybrid particle gene constructs were selected for further purification. The assembly score was based on the dilution of detecting antibody that binds the hybrid particle relative to its binding to wild-type WHcAg. A strong correlation between the relative lysate assembly scores and the ability to purify hybrid core particles in high yield has been observed during development of the present invention. Every  
10 hybrid particle construct with an assembly score of three or greater in the transformed bacterial lysate has yielded easily-purifiable particles. In contrast, constructs with assembly scores of two or less have been problematic to purify.

## V. Antigenic and Immunogenic Characterization of WHcAg-Hybrid Particles

15

### A. Epitope Selection

A group of model epitopes/antigens has been selected for use to further develop the WHcAg platform technology. Three categories of antigens are examined: (1) peptidic epitopes are inserted using recombinant methods; (2) polysaccharide (PS) antigens are chemically conjugated to lysine-modified core particles; and (3) larger or non-linear protein antigens are incorporated onto core particles by recombinant or chemical methods. Selected peptidic epitopes include those shown in Table 9. Selected protein/polypeptide antigens include but are not limited to the *Bacillus anthracis* capsular polypeptide poly-gamma-D-glutamic acid, which has been chemically conjugated to WHcAg (*See, Figure 31*).

25

### B. Epitope Optimization on Hybrid Core Particles

Because the inserted epitope sequence can effect hybrid core assembly or stability, it is useful to perform mutational analysis of the epitope in order to map the necessary antibody contact residues. Non-essential residues are subsequently substituted with other less disruptive residues as needed. This strategy is also useful for identifying analogs with improved antibody binding. The M2e epitope serves as an example of this strategy. A list of M2-WHcAg hybrid

constructs (-IM2 series) with different C-termini and two different insert sites were produced and the relative expression levels and assembly competence scores are shown in Figure 22. Note that all constructs harboring the wild-type M2e sequence either assembled poorly or were purified as aggregates instead of core particles. It is contemplated that the two cysteines in the wild-type  
5 M2e sequence result in inappropriate inter- or intra-particle disulfide bridges. Therefore, an M2e-specific mAb (14C2, which inhibits Influenza A growth of most strains) was tested for binding to a peptide analog panel, including cysteine-substituted peptides as shown in Figure 23. Because substitution of either or both cysteine residues did not effect the binding of the 14C2 mAb, hybrid core constructs carrying the mutated M2e sequence, IM2(-), inserted at position 78  
10 were produced. The IM2(-) sequence expressed in the context of the 150C C-terminus still resulted in aggregates during purification. However, the IM2(-) sequence inserted at position 78 in the context of the HyW-C-terminus allowed assembly and was easily purified (See, Figure 22). Subsequently, other combinations of C-termini and insert positions have been found to accommodate the IM2(-) sequence, such as HyW2-IM2(-)81. These other M2e-WHcAg hybrid  
15 particles have also been tested for immunogenicity. Additional modifications to the M2e epitope are also done to optimize the hybrid WHcAg expression and particle assembly. In some embodiments, substitution of single cysteine residues (C16 and C18) have been made, and multiple copies of a M2e(-) truncated sequence were inserted. Note that P4 (a 15-mer) also bound mAb 14C2 efficiently, as did the polyclonal anti-HyW-IM2(-)78 antisera (See, Figure 23).

20

### C. Antigenicity

Prior to *in vivo* immunogenicity testing all purified hybrid WHcAg particles are characterized for antigen expression at the B cell level by measuring the ability to bind polyclonal or mAbs specific for the WHcAg carrier and the peptidic, protein or PS insert. The  
25 same capture ELISA system used to detect hybrid WHcAg particles in bacterial lysates is used for purified particles. T cell antigenicity is determined by assessing the ability of the hybrid WHcAg particles to activate core-specific T cells *in vitro*. For this purpose naive splenic T cells from T cell receptor (TCR) transgenic (Tg) (7/16-5-TCR) mice which have a high frequency of HBcAg-specific CD4<sup>+</sup> T cells (~50%) are used. The 7/16-5 TCR crossreacts with HBcAg and  
30 WHcAg as it recognizes HBcAg<sub>129-140</sub> presented by IA<sup>b</sup> and this sequence is very similar between HBcAg and WHcAg. After a 2 day culture of 7/16-5-TCR spleen cells with HBcAg/WHcAg,

the IL-2 that is secreted into the supernatant (SN) is measured by ELISA (See, Figures 24 and 25). This is a convenient and rapid screen to check if any of the modifications to the WHcAg protein have disrupted core-specific T cell recognition. Additional WHcAg-specific TCR-Tg lineages that recognize different T cell sites are similarly employed (Chen *et al.*, J.Virol. 74: 5 7587-7599, 2000). In cases when a pathogen-specific heterologous CD4<sup>+</sup> T cell epitope is inserted into the hybrid core particle, mice of the appropriate H-2 haplotype (e.g., high responder) are immunized with the hybrid particle (10 µg, subcutaneously in IFA) and draining lymph node cells are harvested 7-10 days later for culture with the heterologous peptide, as well 10 as a WHcAg-derived peptide panel. T cell activation and specificity is determined by cytokine production (IL-2, IL-4, IFN $\gamma$ ) recalled by the peptide antigen panel. Cytokines are measured in 2 day (IL-2) or 4 day (IL-4, IFN $\gamma$ ) SNs by ELISA.

#### D. Immunogenicity

The immune response to hybrid-WHcAg particles and WHcAg-PS conjugates is examined in detail. In addition to anti-insert or anti-PS and anti-WHcAg antibody end-point titers, antibody specificity, isotype distribution, antibody persistence and antibody avidity are monitored. Examples of these assays are provided below. *In vivo* immune responses to PS-WHcAg conjugates are compared to free PS and to the same PS linked to other protein carriers (e.g., tetanus toxoid). *In vivo* antibody production is studied in inbred murine strains, in athymic 15 mice, in H-2 congenic mice, and in core-specific TCR-Tg mice. The use of these strains permits the evaluation of non-H-2 and H-2 dependent genetic influences on immune responsiveness, as well as T cell independence (athymic mice). In addition, the TCR-Tg mice permit the screening of a number of hybrid particles/conjugates rapidly *in vivo* (e.g., 2-4 weeks), because the kinetics 20 of antibody production to the WHcAg carrier and inserted epitopes is accelerated in these mice.

Carrier-specific and insert-specific Th cell immunogenicity is monitored by assessing T cell activation, fine-specificity and cytokine production. For B cell peptidic epitopes inserted into WHcAg, or PS antigens linked to WHcAg, the source of T cell help is predictably WHcAg-specific Th cells. However, if peptidic T cell sites are inserted into WHcAg or larger protein fragments are incorporated into WHcAg particles, then the source of functional T cell help is not 25 readily apparent. To determine if exogenous T cell sites are functional, mutant core particles with a single substitution at residue 132 (Y132A) have been produced. The tyrosine at position 30

132 represents a dominant aggretopic (MHC-binding) residue in H-2<sup>b</sup> mice, and this alanine substitution converts H-2<sup>b</sup> mice into WHcAg-nonresponders at the Th cell level. Therefore, production of hybrid WHcAg particles containing the Y132A mutation in WHcAg allow the T cell helper function of the exogenous T cell site to be measured as a function of *in vivo* antibody 5 production to the inserted B cell epitope.

#### E. *In Vitro* Correlates of the Immune Response to Hybrid Core Particles

The hepatitis core proteins are extremely immunogenic *in vivo* during natural infection and as immunogens. It is contemplated that *in vitro* correlates of immunogenicity can serve as 10 rapid screening methods to circumvent long term *in vivo* studies.

##### 1. Induction of Costimulatory Molecules and T Cell Activation

*In vitro* culture of naive resting murine B cells with native HBcAg or WHcAg sufficiently crosslinks mIg receptors on core-specific B cells for induction of the costimulatory 15 B7.2 (24 hrs.) and B7.1 (72 hrs.) molecules (Milich *et al.*, Proc Natl Acad Sci, USA, 94:14648-14653, 1997). The HBV envelope particulate antigen (HBsAg) does not demonstrate this property nor do many non-particulate experimental antigens (e.g., hen egg lysozyme, pigeon cytochrome C, etc.). This property is important because it allows naive, resting B cells to become competent APC for primary T cells. Figure 24 illustrates that naive B cells more 20 efficiently present the HBcAg to naive CD4<sup>+</sup> cells derived from 7/16-5-TCR Tg mice than do the more classic APCs, splenic adherent cells (MØ/DC). In contrast, MØ/DC APC present peptide HBcAg<sub>120-140</sub> more efficiently than do B cells. Previously it has been shown that HBcAg-specific B cells are the primary APC in murine spleen cultures (Milich *et al.*, *supra*, 1997), and that there is a high frequency (8%) of HBcAg-binding B cells among naive murine spleen cells 25 (Lazdina *et al.*, J Virol, 75:6367-6374, 2001), as well as in naive human PBL (Cao *et al.*, J Virol, 75:6359-6366, 2001). Preliminary studies illustrate that the WHcAg and hybrid-WHcAg particles containing malaria inserts in the loop are also preferentially presented to T cells by naive splenic B cells (See, Figure 25). To confirm that this B cell APC function is dependent upon induction of B7.1 and B7.2 costimulatory molecules a variety of WHcAg-hybrid particles 30 are cultured with naive resting, splenic B cells over a 72 hour period. Induction of B7.1 and B7.2 mRNA is then measured by RT-PCR, and expression of B7.1 and B7.2 protein is measured

by FACS analysis. WHcAg hybrid particles differing in number of inserts, position of those inserts, and C-termini are compared to identify correlations between structure and induction of B7.2 and/or B7.1. Inhibition of B7.2/B7.1 induction with anti-mIg and anti-insert monoclonal antibodies is contemplated to confirm that B7.2/B7.1 induction is mediated through crosslinking  
5 of the antigen-specific mIg receptor.

## 2. *In Vitro* Primary Antibody Production as an *In Vitro* Model of Immunogenicity

It is contemplated that like T cell activation, that anti-core antibody production is  
10 mediated through core antigen-specific B cells acting as the primary APC source. Indeed, after five days in culture with either HBcAg or WHcAg, spleen cells from 7/16-5 TCR-Tg mice were shown to produce high levels of IgM anti-HBc or anti-WHc (See, Figure 26). Strikingly, the WHcAg induced higher levels of IgM anti-core antibody than did the HBcAg at all antigen concentrations. During development of the present invention, *In vitro* IgM anti-core production  
15 was found to: (1) require core-specific T cells since this response does not occur in non-TCR<sup>+</sup> control splenic cultures; (2) require particulate core antigens since non-particulate HBeAg or WHeAg elicit only low levels of antibody; and (3) be antigen-specific since the IgM anti-HBc and anti-WHc antibodies produced are non-crossreactive. To determine the generality of this finding, a variety of hybrid WHcAg particles containing different inserted epitopes at different  
20 positions with varying C-termini were tested in the *in vitro* IgM antibody production assay using 7/16-5-TCR spleen cells. Indeed, as shown in Figure 27, hybrid core particles did elicit *in vitro* IgM antibody production variably from high levels to no antibody. Importantly, the level of *in vitro* IgM antibody production positively correlated with anti-insert IgG antibody production *in vivo* after primary immunization with hybrid WHcAg particles. Thus, substitution of primary *in*  
25 *vitro* IgM production for *in vivo* immunization is contemplated to be an efficient method for screening large numbers of hybrid WHcAg particles relatively quickly. Interestingly, the IgM antibodies present in the SNs bind to their respective hybrid WHcAg particles better than to native WHcAg, and better than to their respective peptide inserts. Thus, the IgM antibodies are contemplated to recognize a combined structural component on the surface of the hybrid  
30 particles

## F. Functional Characteristics of Immune Responses to Hybrid Core Particles

As many of the heterologous epitopes utilized are engineered or weak peptidic and PS epitopes, it is important to determine the extent to which antibodies induced by hybrid WHcAg particle or core-glycoconjugate immunization recognize the native antigen (preferably in the context of the pathogen). For instance, anti-M2e antibodies elicited by immunization with HyW-IM2(-)78 particles recognize native M2 in Influenza A infected cell cultures (See, Figure 34). In some embodiments when it is not practical to test antibody binding on the pathogen, at least a purified native protein is also tested. For example anti-SEB<sub>140-151</sub> and anti-SEB<sub>152-161</sub> antisera are tested by ELISA on a panel of SEs. Similarly IgG antibody avidity for the native protein is determined. For this purpose, Na SCN is used as a chaotropic agent as previously described (Anttila *et al.*, J Infect Dis, 177:1614-1621, 1998). The basic ELISA is performed with one exception, before the detecting antibody is introduced, 100 µl of 0.5M Na SCN in PBS or PBS alone is added to each microtiter well for 15 minutes at RT. The wells are then washed four times and the ELISA is completed as usual. The results are expressed as avidity indices (e.g., titer with Na SCN/titer without Na SCN) x 100.

### 1. Antibody Function

For example, anti-M2e antisera is assayed for *in vitro* plaque size reduction (See, Figure 35), as a measure of antibody function. Another example involves an analysis of antibodies reactive with SEB (a super antigen for Vβ8<sup>+</sup> T cells). Both the SEB<sub>140-151</sub> and SEB<sub>152-161</sub> peptides and antibodies have been shown to inhibit human Vβ8+ T cell activation *in vitro* (Arad *et al.*, Nat Med, 6:414-421, 2000; and Visvanathan *et al.*, Infect Immunol, 69:875-884, 2001). Similarly, passive transfer of anti-SEB<sub>152-161</sub> and anti-SEB<sub>140-151</sub> antisera have been shown to protect mice against a lethal intra-peritoneal challenge with SEB and LPS. Although mice are not very sensitive to SE-induced toxic shock, Balb/c mice primed with D-galactosamine (20 mg) followed by low dose LPS (1-10 ng) become extremely sensitive to SEB (20 ng) and exhibit 100% lethality (Visvanathan *et al.*, *supra*, 2001). Antisera generated by immunization with HyW2-SE75 is tested in these two functional assays. The host defense against many bacterial pathogens depends on the opsonic activity of anti-PS antibodies, the complement pathway, and phagocytosis by macrophages. Opsonic activity of mouse antisera to WHcAg-PS conjugates is determined in an opsonophagocytosis assay as described (DeVelasco *et al.*, Vaccine, 12:1419-

1422, 1994). Fluorescein-labelled bacteria are opsonized with dilutions (0-20%) of heat-inactivated mouse antisera in the presence or absence of complement (2%). Anti-PS antibodies are neutralized prior to performing the assay. The mouse macrophage cell line RAW-264 ( $10^7$  cells/ml) is used for phagocytosis of bacteria at a bacteria/macrophage ratio of 10:1.

5 Macrophages are washed and analyzed by flow cytometry. The mean of the FITC-intensity of the cells in each sample is used to estimate the opsonic capacity of the antisera.

## 2. Immunization and Challenge Studies

Once hybrid WHcAg particles or WHcAg-PS conjugates have been optimized for 10 immunogenicity and antibody function *in vitro* or via passive transfer of immune sera, immunization/challenge experiments are performed, dependent upon a number of factors. For example, the availability of an infectious model system, the biosafety level of the pathogen, and the appropriate collaborations with disease model experts. In one embodiment, challenge experiments are done using the Influenza A system.

15

## VI. Expansion of the WHcAg Platform to Accommodate Non-Linear, Large Protein and Carbohydrate Epitopes

### A. Construct Design for Expression of Mosaic Core Particles

20 The WHcAg platform technology is also applicable to non-linear larger domains or protein fragments. Insertion of larger amino acid sequences is advantageous not only for presentation of a larger number of epitopes, but also to allow appropriate folding of conformational epitopes. Attempts to obtain stable large protein hybrid HBcAg particles have failed (Koletzki *et al.*, J Gen Virol, 78:2049-2053, 1997), with the single exception of the entire 25 green fluorescence protein inserted into the loop of the HBcAg (Kratz *et al.*, Proc Natl Acad Sci USA, 96:1915-1920). In addition to the molecular adjuvants described below, the non-toxic C fragment of tetanus toxoid (TTFC) is used as a model protein antigen. Since challenge with tetanus toxoid (TT) is well established in the mouse model, expression of TTFC is used to evaluate the ability of a vaccine vector to elicit a protective antibody response. Recombinant 30 particles with TTFC inserted at the N- or C-terminus or into the loop region of WHcAg are produced. In some embodiments, the incorporation of TTFC into core particles is accomplished

by production of mosaic TTFC-WHcAg particles. Specifically, decreasing the number of large foreign sequences per hybrid WHcAg particle to be co-incorporated with unmodified WHcAg protein subunits is contemplated to overcome any steric hindrance. The following approach has been shown to be effective (Smiley and Minion, Gene, 134:33-40, 1993) and is compatible with 5 the cloning and expression vectors (pUC, and pET11d) described herein, although other approaches are also suitable.

### **1. Co-Expression of Wild-Type and WHcAg Fusion Proteins Mediated by a Suppressor tRNA-Readthrough of a Stop Codon**

10 As shown in Figure 28, oligonucleotides are generated to possess a TGA stop codon, as well as the coding information for an additional five amino acids predicted to form a coil secondary structure (Gly5). The oligonucleotides are annealed and the resulting duplex is inserted between the wild-type WHcAg and the fused protein (*e.g.*, WHcAg-TTFC). The derivative plasmid (coding for the fusion WHcAg protein) is used to transform the *E. coli* K12 15 K802 strain or others bacterial strains that possesses an opal TGA-Trp suppressor tRNA under lac repressor control as one example (Smiley and Minion, *supra* 1993). This approach results in the co-expression of both the wild-type WHcAg protein (HyW) and the fusion-WHcAg protein (HyW-TTFC) in the same bacterial cell. To design the fusion-WHcAg core protein, several of the C-terminal modifications are tested to identify those that favor the expression/assembly of a 20 mosaic core particle.

### **2. Co-Expression of Wild-Type and WHcAg Fusion Proteins by Using Differentially Inducible Plasmids**

As shown in Figure 29, a second approach is taken which utilizes two plasmids 25 differentially-induced to express the wild-type and the fusion-WHcAg proteins. In some embodiments, the constructs have been made in pUC18 as the cloning and expression vector, permitting IPTG-inducible expression due to the presence of the Lac promoter. For convenience, only the gene coding for the wild-type WHcAg is subcloned into the pLEX expression vector. The pLex expression vector contains the strong P<sub>L</sub> promoter to drive the 30 expression of the gene of interest (*e.g.*, wild-type WHc gene). The P<sub>L</sub> promoter is controlled by the lambda cl repressor protein, which is expressed in the *E. coli* host (G1698 strain). The cl

repressor was engineered into the bacterial chromosome under control of the tightly regulated *trp* promoter. The expression of the gene is induced by addition of tryptophan thereby suppressing the synthesis of the *cI* repressor. Therefore, the same *E. coli* (G1698) is co-transformed with pUC encoding the fusion-WHcAg protein and with pLEX encoding the wild-type WHcAg protein. The induction of expression of the proteins is then induced differentially by using IPTG and Tryptophan.

### B. Traditional and Molecular Adjuvants

Although adjuvants are not required when using the WHcAg delivery system, some 10 embodiments of the present invention employ traditional and/or molecular adjuvants. Specifically, immunization in saline effectively elicits anti-insert antibody production. However, formulation in non-inflammatory agents such as IFA (mineral oil), Montanide ISA 720 (squalene), and aluminum phosphate ( $\text{AlPO}_4$ ), enhance immunogenicity (See, Figure 30, Panel A). Additionally, administration of WHcAg results in the production of all four IgG isotypes, 15 regardless of which if any adjuvant is employed (See, Figure 30, Panel B). Inclusion of a CpG motif also enhances the primary response. Moreover, use of an inflammatory adjuvant such as the Ribi formulation is not more beneficial than is the use of non-inflammatory adjuvants, indicating that the benefits of the adjuvants result from a depot effect rather than from non-specific inflammation. Thus, the core platform is used with no adjuvant or with non- 20 inflammatory adjuvants depending upon the application and the quantity of antibody desired. In some embodiments of the present invention, IFA is used in murine studies, whereas alum or squalene is used in human studies.

In instances where it is desirable to deliver hybrid WHcAg particles in a single dose in saline (e.g., a nasal influenza A M2e-core post-exposure vaccine), a molecular adjuvant is 25 employed. A number of molecular adjuvants are employed to bridge the gap between innate and adaptive immunity by providing a co-stimulus to target B cells or other APCs. For this purpose in some embodiments, the complement C3d fragment (GenBank Accession No. NM 009778) is employed, as two or three copies of C3d linked to the experimental antigen hen egg lysozyme (HEL) was shown to be three to four orders of magnitude more immunogenic than HEL alone 30 (Dempsey *et al.*, *Science*, 271:348-350, 1996), even in the absence of a traditional adjuvant. C3d targets antigen to B cell and follicular dendritic cells via binding to CD21, thereby costimulating

B cells through its association with CD19, a B cell membrane protein that amplifies B cell activation (Tedder *et al.*, *Immunol Today*, 15:437-442, 1994).

Similarly, soluble dimeric or trimeric forms of CD40L (GenBank Accession No. X65453) have been shown to bind and cross-link membrane CD40 sufficiently to induce B cell proliferation, costimulate Ig class switching, suppress B cell apoptosis and activate APC (Morris *et al.*, *J Biol Chem*, 274:418-423, 1999). Additional potential molecular adjuvants include but are not limited to: i) soluble BAFF (B cell activating factor belonging to the TNF family; GenBank Accession No. AF119383), which exclusively binds to B cells and functions as a potent B cell growth factor (Mackay and Browning, *Nature Reviews Immunology*, 2:465-475, 2002), ii) soluble LAG-3 (lymphocyte activation gene-3; GenBank Accession No. NM 008479), which binds MHC class II molecules with high avidity and elicits activation/maturation of dendritic cells (ElMir and Triebel, *J Immunol*, 164:5583-5589, 2000), and iii) immunostimulatory CpG oligodeoxynucleotides, which costimulate a variety of immune cells (Krieg *et al.*, *Nature*, 374:546-549, 1995). In some embodiments, these molecules are linked to the C-terminus of hybrid core particles to activate only the antigen-specific B cell or APC that takes up the particle, as opposed to the non-specific activation induced by merely mixing the adjuvants with antigen. Less than 100% substitution is desirable because of potential negative effects on particle assembly and/or overstimulation of the targeted cell. Therefore, in some embodiments, mosaic hybrid core particles carrying fewer adjuvant molecules are produced.

20

### 1. Inclusion of CpG Dinucleotides in Hybrid Core Particles

Unmethylated CpG dinucleotides have been shown to be potent immune activators of B cells and macrophages (Krieg *et al.*, *supra*, 1995; and Davis *et al.*, *J Immunol*, 160:870-876, 1998). Additionally, co-immunization of antigen and CpG dinucleotides (DN) enhances the immune response similar to traditional adjuvants. Two characteristics of the CpG effect include: i) B cell uptake is required for activation; and ii) the CpG motif preferentially activates B cells that simultaneously encounter their specific antigen. Given the non-specificity of the effects (e.g., all B cells will internalize CpG DN), large quantities of CpG DN are necessary *in vivo*.

Hybrid core-CS(NANP) particles are a very efficient method for delivery of CpG DN to the interior of antigen-specific B cells. A core-CpG DN complex directly binds to core-specific B cells, and B cell activation occurs due to crosslinking of the mIg receptor and simultaneous

delivery of the CpG DN to the B cell interior. This permits efficient, selective delivery of CpG DN to activated, antigen-specific B cells (e.g., NANP-specific B cells in the case of core-CS hybrid particles). Indeed, preliminary data indicate that HBcAg or HBcAg-CS hybrid particles carrying bacterial RNA/DNA are more immunogenic than particles devoid of *E. coli*-derived RNA/DNA. Full-length HBcAg possesses a RNA/DNA binding sequence at the C-terminus, which is lost upon truncation at residue 149. Unmethylated CpG DNs are much more frequently found in bacterial DNA than in vertebrate DNA. As shown in Table 5, full-length HBcAg<sub>183</sub> is significantly more immunogenic than truncated HBcAg<sub>149</sub> when limiting doses (0.2 µg) are injected in saline in the absence of a traditional adjuvant. Unexpectedly, this difference in immunogenicity is abolished when an adjuvant is used (e.g., CFA).

**Table 5. Bacterial Nucleic Acid Augments the Immunogenicity of HBcAg Particles<sup>1</sup>**

Immunogen	Dose (µg)	RNA DNA	Anti-HBc Titer				Anti-NANP Titer			
			G <sub>1</sub>	G <sub>2a</sub>	G <sub>2b</sub>	G <sub>3</sub>	G <sub>1</sub>	G <sub>2a</sub>	G <sub>2b</sub>	G <sub>3</sub>
HBcAg <sub>183</sub>	0.2	+	0	40,960	40,960	650				
HBcAg <sub>149</sub>	0.2	-	0	0	640	0				
HBcAg <sub>183-</sub> NANP	1.0	+	10,240	2,560	10,240	640	160	160	10,240	640
HBcAg <sub>149-</sub> NANP	1.0	-	0	160	640	0	40	40	160	160

<sup>1</sup>Balb/c mice were immunized with the indicated dose of full-length HBcAg (183 amino acids), truncated HBcAg (149 amino acids), or full-length or truncated HBcAg containing

15 (NANP)<sub>4</sub> inserts in the loop region suspended in saline. Serum was collected four weeks after primary immunization and analyzed by IgG isotype-specific ELISA.

The positive effect of bacterial RNA/DNA was also observed when full-length versus truncated HBcAg-CS hybrid particles were used, resulting in significantly higher anti-NANP 20 antibody production. Synthetic CpG DNs are first chemically coupled to WHcAg or WHcAg-

CS hybrid particles. For this purpose, CpG DNA is modified to contain 5' amino groups, which are subsequently used to conjugate the oligonucleotides to WHcAg particles. In other embodiments, recombinant methods are used to incorporate CpG motifs into the interior of WHcAg using nucleic acid-binding motifs. Internalization of the CpG sequence is expected to  
5 reduce its sensitivity to nucleases. The effects of inclusion of CpG DN into hybrid WHcAg particles is determined by immunization of hybrid particles with and without CpG DN, by comparing the anti-insert and anti-WHcAg humoral responses and the WHcAg-specific Th cell responses in various *in vivo* assays. CpG DN-coupled hybrid particles are also used in various TCR-Tg splenic *in vitro* assays of cytokine production and IgM antibody production.

10

## 2. Other Molecular Adjuvants

Genes encoding the murine CD40L (both 655 and 470 nucleic acid versions) have been used successfully to express these ligands at the C-terminus of WHcAg (See, Figure 38). Moreover, immunization of mice with hybrid WHcAg-CD40L particles results in the production  
15 of higher anti-core antibody titers than does the immunization of mice with WHcAg particles (See, Figure 39). However, lower than desirable yields of purified particles have been obtained. Therefore, mosaic particles containing less than 100% CD40L-fused polypeptides are produced to overcome this problem.

The other molecular adjuvants inserted within the WHcAg, including the C3d fragment,  
20 BAFF and LAG-3, have a tendency to become internalized when inserted at the C-terminus. Therefore tandem repeats of molecular adjuvants are used to resist internalization. Alternatively, various mutations within the so-called hinge region of WHcAg, between the assembly domain and the DNA/RNA-binding region of the core particle are made to prevent internalization of C-terminal sequences. However, internalization represents a problem only for those molecular  
25 adjuvants such as CD40L, C3d, BAFF and LAG-3, which function at the APC/B cell membrane. In contrast, internalization of molecular adjuvants such as CpG DN is not an issue as these types of adjuvants function at the level of cytosolic receptors.

### C. Chemical Coupling of Protein and Carbohydrate Antigens

In those instances when it is not possible to incorporate large protein epitopes or molecular adjuvants into the WHcAg by recombinant methods, chemical conjugation is used. Similarly, the WHcAg also serves as a new type of carrier platform for polysaccharide or 5 oligosaccharides (PS/OS) antigens upon chemically coupling PS/OS epitopes to the WHcAg core.

#### 1. Modification of Core Genes for Subsequent Chemical Conjugation

The wild type WHcAg is not efficiently chemically derivatized. Therefore, reactive 10 amine groups are added by the insertion of one to several lysines via recombinant technologies. The position and number of the added lysines is varied (e.g., N- and C-termini, and within or outside the exposed loop region).

#### 2. Model Protein Antigen

One protein and two carbohydrate model antigens are used for testing the feasibility of 15 chemical conjugation to lysine-modified WHcAg. The model protein antigen, Poly-gamma-D-glutamic acid (PGA), is the capsular polypeptide of *Bacillus* sp. including *B. anthracis* (Fouet et al., J Appl Microbiol, 87:251-255, 1999). The capsular PGA of anthrax pathogens is very similar to bacterial cell surface PS antigens in that they are both poorly immunogenic, repetitive 20 polymers require coupling to a carrier moiety. To produce WHcAg-PGA conjugates, a carbodiimide-mediated coupling reaction is used because PGA molecules contain abundant carboxylate groups. The cores initially employed for this purpose include WHcAg-HyW2 and WHcAg-HyW2 modified with a lysine insert within the loop (K<sup>75</sup>) or at the C-terminus. In 25 preliminary studies using WHcAg-HyW2 and a saturation coupling approach, equal amounts of PGA and WHcAg-HyW2 (1.3 mg) and 5.0 mg of carbodiimide were mixed and after a four hour reaction time, SDS-PAGE analysis revealed that most of the PGA was coupled to WHcAg. As shown in Figure 31, immunization of Balb/c mice with 10 µg of the PGA-WHcAg-HyW2 conjugate formulated in alum resulted in production of significant IgM and IgG anti-PGA antibodies, whereas the uncoupled PGA in alum was non-immunogenic. Note that IgM anti- 30 PGA decreased from the first immunization to the third and IgG anti-PGA antibodies increased from the first immunization to the third. In other embodiments, the lysine-modified WHcAg

particles are used with various PGA polymer sizes and WHcAg/PGA ratios, to optimize conjugate production and immunogenicity. The WHcAg carrier is expected by the inventors to compare favorably with common toxoid carriers (e.g., tetanus toxoid and diphtheria toxoid). *In vitro* opsonophagocytic assays (DeVelasco *et al.*, Vaccine, 12:1419-1422, 1994) are used to test  
5 the function of anti-PGA antibodies. As a surrogate for *B. anthracis*, *B. licheniformis* 9945A (not a human pathogen) which has the same PGA capsule is used. In some embodiments, the ability of the PGA-WHcAg conjugate to protect immunized mice against lethal *B. anthracis* challenge is assessed.

10 **3. Model Carbohydrate Antigens**

The O-antigenic PSs are both essential virulence factors and protective *Shigella* antigens. Moreover, serum IgG specific for O-PS has been demonstrated to confer immunity against *shigellosis*. Despite these findings, to date no licensed *Shigella* vaccines exist. To meet this need in the art, a lysine-modified WHcAg is used as a carrier platform for the O-PSs of *Shigella*,  
15 with a particular focus on *S. dysenteriae* 1 and *S. flexneri* 2a. A recent study using recombinant core protein derived from the duck hepatitis virus coupled to purified type III capsular PS from group B streptococcus (GBS) demonstrated 97% survival after GBS type III challenge in newborn pups born to vaccinated mouse dams (Paoletti *et al.*, Vaccine, 20:370-376, 2002). A method is used that permits the chemical synthesis of an array of glycoconjugates containing  
20 saccharide antigens of desired molecular sizes and that employs chemically controlled site-specific coupling (Wang *et al.*, Vaccine, 21:1112-1117, 2003). Utilizing these chemical methods, particulate PS-WHcAg conjugates are prepared incorporating PS epitopes of known molecular size and orientation which are linked at specified sites to core particles. The size of OS antigens deserves particular attention in the context of using WHcAg as the carrier. The  
25 spacing of natural WHcAg B-cell epitopes and of peptidic antigens inserted in the tips of the spikes is contemplated to be an important determining factor for immunogenicity. Therefore, it is important to test a range of sizes of OS antigens beginning with sizes comparable to peptidic antigens (e.g., 2,000-3,000 kDa), which exhibit high levels of immunogenicity. In addition to coupling large numbers of a single PS epitope to a single particle, the multivalency of the  
30 particles provides the opportunity to couple PS epitopes from many different serotypes to the same particle yielding a multivalent vaccine. Alternatively, particles conjugated with one PS

serotype are mixed with other conjugated particles carrying a different serotype PS. The WHcAg is contemplated to be superior to commonly used carrier proteins for delivery of OS/PS antigens.

5           **VII. Applications of the WHcAg Combinatorial Technology**

A. Infectious Diseases

Historically the use of the HBcAg as a platform has been confined to use as a T cell carrier for neutralizing epitopes of infectious disease pathogens. Subunit vaccine development 10 for infectious diseases remains an important application for the WHcAg platform technology. In one embodiment, the *P. falciparum* CS repeat epitope NANPNVDP(NANP)<sub>3</sub> (SEQ ID NO:75) was inserted in many positions within the WHcAg, and complemented with diverse C-termini as a model system to further develop the WHcAg as a vaccine platform (See, Figures 2, 14 and 15).

15           **1. *Plasmodium Vivax***

This *P. vivax* malaria species is predominant in South and Central America and is also found in Southeast Asia. A bivalent WHcAg hybrid particle was produced containing the *P. falciparum* CS repeat at the N-terminus, and the Type I variants of the *P. vivax*-CS repeat as an insertion at position 78. Preliminary data indicate that the vaccine candidate is effective and that 20 antibodies to both inserts were produced. Thus, the present invention also provides bivalent vaccines based upon the WHcAg technology (See, Figure 15). Since the *P. vivax* system is more complex (because several genotypes exist), more than one vaccine particle is produced and tested. Alternatively, several genotype-specific B cell epitopes are inserted into the same particle at different sites to produce a bivalent or trivalent vaccine particle. For instance, the Type II and 25 Type III variants of the *P. vivax* CS repeat epitopes (See, Table 6) are inserted into the WHcAg platform in addition to the Type I variant:

**Table 6. *Plasmodium vivax* Circumsporozoite (CS) Sequences**

Type	Sequence <sup>1</sup>	Identifier
I	DRAAGQPGDRADGQPG	SEQ ID NO:74
II	ANGAGNQPGANGAGDQPG	SEQ ID NO:65
II	ANGADNQPGANGADDQPG	SEQ ID NO:66
III	APGANQEGGAAAPGANQEGGAA	SEQ ID NO:67

<sup>1</sup>Bold type denotes variant residues.

## 2. Foot and Mouth Disease (FMDV)

One of the first examples of the use of the HBcAg as a vaccine carrier was for the major immunogenic B cell epitope of the FMDV<sub>141-160</sub>. Previously, a hybrid HBcAg-FMDV particle was shown to elicit protective antibodies (Bittle *et al.*, *Nature*, 298:30-35, 1982), although there were problems eliciting sufficiently high levels of anti-FMDV antibodies. The WHcAg combinatorial technology described herein is contemplated to provide a more effective vaccine candidate. Two protective linear epitopes derived from the VP1 protein have been defined: VP1<sub>141-160</sub> and VP1<sub>200-213</sub> (Van Lierop *et al.*, *Immunol*, 75:406-413, 1992). Recently a DNA vaccine encoding VP1<sub>141-160</sub> and VP1<sub>200-213</sub> was shown to protect swine from a FMDV challenge (Wong *et al.*, *Virol*, 278:27-35, 2000). Thus, both neutralizing epitopes are selected for incorporation into hybrid WHcAg particles.

## 3. Influenza A Virus

The extracellular domain of the matrix 2 (M2e) sequence of influenza A has also been chosen as a model neutralizing B cell epitope for insertion within WHcAg, as this sequence has a number of features in common with the malaria CS repeat. The M2e sequence is a linear protective epitope that is poorly immunogenic during natural infection and in the context of various vaccine formulations. Additionally, the M2e sequence permits the comparison of the WHcAg and HBcAg vaccine platforms (Jegerlehner *et al.*, *Vaccine*, 3104, 2002; Neirynck *et al.*, *Nat Med*, 5:1157-1163, 1999; and Heinen *et al.*, *J Gen Virol*, 83:1851-1859, 2002). The kinetics of the antibody response elicited by a M2e-WHcAg hybrid particle (HyW-IM2(-)78) is shown in

Figure 32. Anti-WHc and anti-M2e antibodies are detected within 2 weeks of primary immunization, with serum titers reaching a plateau four weeks after the primary immunization, and rising approximately 10-fold after boosting. Although, an anti-M2e titer of  $3 \times 10^6$  after two doses is two orders of magnitude higher than the levels previously obtained using the HBcAg platform , improvements on the immunogenicity of the HyW-IM2(-)78 particle are contemplated. The sera from mice immunized with HyW-IM2(-)78 were analyzed to determine the isotype distribution of anti-2Me and anti-WHc antibodies. As shown in Figure 33, the response was well represented by all the IgG isotypes. After the second immunization, anti-M2e serum titers greater than 1:100,000 were obtained for all four IgG isotypes. A similar pattern was observed in the anti-WHc response with the exception of a relatively low IgG<sub>3</sub> response to the carrier. The broad spectrum IgG isotype profile specific for the M2e epitope is a positive characteristic, which guarantees a full spectrum of biologic effector functions (complement fixation, ADCC, etc.). Importantly, the HyW-IM2(-)78 antisera also binds to viral M2 and inhibits influenza virus growth.

Quantitating the reactivity of sera from WHcAg-M2e immunized mice against authentic M2 protein is the first step in characterizing WHcAg-M2e as a vaccine candidate. The use of flow cytometry against virus-infected cells is performed on live, unfixed cells thereby ensuring the sera can recognize M2 in its native conformation in the plasma membrane (Pekosz and Lamb, J Virol, 73:8808-8812, 1999). Sera from WHcAg-M2e immunized mice (diluted 1:100) were incubated at 4° C with cells infected with influenza A virus for 12 hours. The samples were washed, incubated with FITC-conjugated goat IgG recognizing mouse IgG, washed and analyzed by flow cytometry. Sera from mice immunized with core particle alone did not specifically react with influenza A virus infected cells in comparison to mock-infected cells (See, Figure 34, panel A). In contrast, sera from WHcAg-M2e immunized mice recognized influenza A virus infected cells, as judged by the shift to increased fluorescence intensity displayed in the virus-infected cell population (See, Figure 34, panel B). Thus, the hybrid particle generates a physiologically relevant antibody response recognizing influenza A virus infected cells.

Certain monoclonal antibodies targeting the M2 extracellular domain restrict virus replication *in vitro* by inhibiting virus particle budding (Hughey *et al.*, Virol, 212:411-412, 1995). The ability of WHcAg-M2e immunized mouse sera to inhibit influenza A virus budding was assayed as described (Zebedee and Lamb, Proc Natl Acad Sci USA, 86:1061-1065, 1989).

Influenza A virus strain rWSN (a M2 monoclonal antibody resistant strain), produced equivalent numbers of infectious particles irrespective of the presence of anti-WHcAg-M2e sera. In contrast, rWSN M-Udorn reassortant virus possessing an RNA segment 7 from a/Udorn/72, but all other segments derived from rWSN (a M2 monoclonal antibody sensitive strain) consistently 5 produced less infectious virus particles at all time points tested when WHcAg-M2e anti sera was present (See, Figure 35). The reduction in virus titer indicated that sera from WHcAg-M2e immunized mice has the ability to inhibit the production of infectious influenza A virus particles *in vitro*. Immunization/challenge studies are also done to assess the ability of the WHcAg-M2e particles to provide protection against influenza A infection.

10 Previously, the HBcAg has been used as a platform for the M2e epitope by positioning the M2e region at the NH<sub>2</sub>-terminus of HBcAg. The first study reported relatively low serum anti-M2e titers ( $4 \times 10^4$ ) after three doses of hybrid HBcAg particles in a strong adjuvant system (Neirynck *et al.*, Nat Med, 5:1157-1163, 1999). Nonetheless, this level of anti-M2e was sufficient to significantly protect mice against a lethal challenge. Another group using the same 15 hybrid HBcAg vaccine candidate in pigs raised less serum anti-M2e antibody ( $3 \times 10^3$ ), and failed to achieve protection. Similarly, a murine study in mice using hybrid HBcAg particles without adjuvant achieved very low anti-M2e serum titers (1:80) and challenged mice were not protected. However, mice receiving a chemical conjugate were protected which correlated with higher anti-M2e serum titers (1:5,120). Thus, it is contemplated that the quantity of protective 20 antibody produced is important and that a threshold serum level is necessary and should be maintained for antibody-mediated protection. As described herein, the M2e sequence was inserted within the loop of WHcAg, and this prototype M2e-WHcAg particle was found to elicit 100-fold more anti-M2e serum antibody ( $3 \times 10^6$ ), than the HBcAg-M2e particles shown in Table 7, even after fewer doses in IFA.

**Table 7. Hybrid Core Particles Containing the Influenza Virus M2e Sequence**

Particle	Dose (adjuvant)	Antibody Titer (1/dilution)		Comment (reference)
		Anti-M2e	Anti-Core	
M2e-HBc	3 (Ribi)	$4 \times 10^4$	$2.7 \times 10^6$	protection (Neirynck, <i>supra</i> , 1999)
M2e-HBc	3 (adjuvant)	$3 \times 10^3$	-	no protection (Heinen, <i>supra</i> , 2002)
M2e-HBc	2 (no adjuvant)	80	-	no protection (Jegerlehner, <i>supra</i> , 2002)
HyW-IM2(-)78	2 (IFA)	$3 \times 10^6$	$3 \times 10^6$	<i>in vitro</i> neutralization (present invention)

5           **4. Anti-Toxin Vaccine Design**

Another suitable application of the WHcAg platform technology is as a toxin subunit vaccine. One advantage of using the WHcAg platform is that a neutralizing epitope of the toxin is inserted into the particles. This is contemplated to be more immunogenic than the whole toxin or toxoid, while circumventing the expense and hazard of dealing with the whole toxin.

10          As a prototype, two peptidic B cell epitopes from Staphylococcal enterotoxin B (SEB) were selected. Anti-SEB<sub>152-161</sub> antibodies recognize native SEB, as well as other SE's and inhibit transcytosis of SEB, SEA, SEE and TSST-1 (Arad *et al.*, Nat Med, 6:414-421, 2000). To prevent SE-mediated disease, a hybrid core particle vaccine has been constructed by inserting the SEB<sub>152-161</sub> sequence in WHcAg at position 75, in combination with the HyW2 C-terminus.

15          Similarly, the SEB<sub>140-151</sub> peptide and antibody have also been shown to be biologically active and highly conserved (Visvanathan *et al.*, Infect Immunol, 69:875-884, 2001). Therefore, the SEB<sub>140-151</sub> sequence is used as well, to produce WHcAg-SEB<sub>140-151</sub> insert particles.

## B. Therapeutic Autoantibodies

The ability of the WHcAg platform to raise very high levels of anti-insert antibody is contemplated to be useful for a number of applications beyond the infectious disease setting. One such application is for the production of therapeutic autoantibodies. Several mAb-based therapies have shown encouraging results in small animal studies and in clinical trials. For instance, mAb therapy targeting c-erbB2 (HER 2/neu) has been used to treat breast cancer (Pegram and Slamon, *Semin Oncol*, 27:13-19, 2000); antibody to  $\beta$ -amyloid has been used to treat an Alzheimer's-like disease in mice (Schenk *et al.*, *Nature*, 400:173-177, 1999), anti-IgE mAb has been tested to treat allergy (Cheng, *Nat Biotechnol*, 18:157-162, 2000), and in human clinical trials an anti-TNF $\alpha$  mAb therapy reduced the symptoms of rheumatoid arthritis and Crohn's disease (Maini and Taylor, *Annu Rev Med*, 51:207-229, 2000). However, active immunization has a number of advantages over passive mAb therapy: i) patient convenience and cost (several immunizations as opposed to numerous infusions, each requiring several hours in the clinic); ii) costs for large scale mAb production are extremely high; iii) active immunization produces more consistent levels of antibody over time; and iv) mAb therapy is likely to induce an inactivating antibody response. In fact, others have begun using Papillomavirus-like-particles chemically conjugated to self antigens to elicit therapeutic autoantibodies (Chackerian *et al.*, *J Clin Invest*, 108:415-423, 2001; and Chackerian *et al.*, *Proc Natl Acad Sci USA*, 96:2373-2378, 1999). Thus, several model epitopes in the context of WHcAg are used to induce production of autoantibodies possessing therapeutic functions.

### 1. Anti-CETP Autoantibody

There is a strong inverse relationship between the plasma concentration of cholesterol in HDLs and the development of coronary heart disease. One therapeutic approach that has been suggested for increasing HDL concentrations is the inhibition of cholesteryl ester transfer protein (CETP) activity (Tall, *J Lipid Res*, 34:1255-1274, 1993). The CETP functions in the plasma to lower HDL by moving cholesteryl esters from HDLs to VLDLs and LDLs (Barter *et al.*, *Biochem J*, 208:1-7, 1982). Transient inhibition of CETP activity in rabbits and hamsters by mAb, small molecules, or antisense oligonucleotides (Whitlock *et al.*, *J Clin Invest*, 84:129-137, 1989; Kothari *et al.*, *Atherosclerosis*, 128:59-66, 1997; and Sugano and Makino, *J Biol Chem*, 271:19080-19083, 1996) causes an increase in plasma HDL. In addition, sustained inhibition of

CETP expression by antisense oligonucleotides increased plasma HDL and reduced atherosclerotic lesions in rabbits (Sugano *et al.*, J Biol Chem, 273:5033-5036, 1998). In contrast, transgenic mice and rats expressing human CETP have decreased plasma HDL (Agellon *et al.*, J Biol Chem, 266:10796-10801, 1991; and Herrera *et al.*, Nat Med, 5:1383-1389, 1999). Similarly, 5 human populations with reduced or absent CETP activity due to genetic mutations have markedly elevated plasma HDL (Koizumi *et al.*, Atherosclerosis, 58:175-186, 1985). Recently a vaccine approach was used to generate antibodies against CETP *in vivo* in rabbits using a dominant linear B cell epitope consisting of residues 461-476 of human CETP. The immunized rabbits had reduced CETP activity, a substantial increase in HDL, and a significant reduction in 10 aortic atherosclerotic lesions (Rittershaus *et al.*, Arterioscler Thromb Vasc Biol, 20:2106-2112, 2000). For this reason, the CETP<sub>461-476</sub> sequence was inserted within the WHcAg platform at position 74 in combination with the HyW C-terminal platform (HyW2-CETP<sub>74</sub>). Interestingly, attempts to insert the CETP sequence at positions 75 or 78 were unsuccessful illustrating the 15 versatility of the WHcAg combinatorial technology. As shown in Figure 36, significant levels of anti-CETP<sub>461-476</sub> antibodies were raised in mice immunized with HyW2-CETP<sub>74</sub>, and secondary anti-CETP<sub>461-476</sub> antibodies also neutralize CETP activity *in vitro*. Because mice do not express CETP, this does not represent an autoantibody response. However, immunizing Tg mice 20 expressing human CETP (and having lowered HDL levels) with HyW-CETP<sub>74</sub> particles is contemplated to induce anti-CETP antibodies capable of both neutralizing CETP activity and raising plasma HDL levels *in vivo*. The ability of immunization with the CETP<sub>461-476</sub> peptide linked to a tetanus toxoid peptide to raise HDL levels in vaccine recipients is currently being assessed in human clinical trials (Rittershaus *et al.*, *supra*, 2000). A particulate WHcAg-CETP<sub>461-476</sub> vaccine is contemplated to be a superior immunogen in humans as compared to a totally peptidic vaccine.

25

## 2. Anti-Cytokine Autoantibody

In many autoimmune diseases such as multiple sclerosis and diabetes, Th cells play a predominant role. The Th cells function both to help B cells produce antibody (Th<sub>2</sub> cells) and to mediate inflammatory responses (Th<sub>1</sub> cells). Th<sub>1</sub> cells mediate their inflammatory function 30 through the production of cytokines, which have direct anti-pathogen effects, as well as effects on other immune cell types. Tumor necrosis factor-alpha (TNF $\alpha$ ) is an inflammatory cytokine

that is often produced during autoimmune reactions and is responsible for many of the lesions or symptoms. Therefore, B cell epitopes from TNF $\alpha$  are inserted into the WHcAg platform and the effect of high levels of anti-cytokine (e.g., anti-TNF $\alpha$ ) antibodies is assessed in animal models of ongoing autoimmune disease. Analogues of TNF $\alpha$  and soluble receptors for TNF $\alpha$ , as well as 5 anti-TNF $\alpha$  mAbs are currently tested with promising results in clinical trials. However, these drugs are very expensive and require continual dosing, whereas anti-TNF $\alpha$  antibodies produced by the patient following vaccination are inexpensive and work via the same TNF $\alpha$ -blocking mechanism. The murine TNF $\alpha_{3.22}$  epitope is used as a model antigen because this region is homologous to human TNF $\alpha$ , has been reported to interact with the TNF $\alpha$  receptor and in a 10 mouse model vaccination with this TNF $\alpha$  peptide chemically conjugated to Papillomavirus-like particles produced anti-TNF $\alpha$  autoantibodies and inhibited development of type II collagen-induced arthritis (Chackerian *et al.*, J Clin Invest, 108:415-423, 2001). WHcAg-TNF $\alpha_{3.22}$  hybrid particles are produced and their immunogenicity is examined using methods disclosed herein. Moreover, the effects of anti-TNF $\alpha$  autoantibody production on the induction and/or 15 maintenance of type II collagen-induced arthritis in a mouse model is assessed.

Although the delivery of self B cell epitopes on a particulate structure can circumvent T cell self-tolerance and elicit autoantibodies, this is a more complicated system than simply eliciting antibodies to foreign epitopes. Various properties of this system are examined using HBcAg- and HBeAg-Tg mice and T cell receptor (TCR) double-Tg mice. For instance, 20 autoantibodies were elicited (anti-HBe/anti-HBc) in TCR x HBe/HBc-double Tg mice by the single injection of a peptide that activates the HBe/HBcAg-specific transgenic CD4 $^+$  T cells (See, Figure 37). Note that anti-HBe autoantibody production is transient lasting approximately three months. In HBeAg-Tg mice expressing a higher serum level of HBeAg, autoantibody production is even more transient (e.g., 2-3 weeks). In contrast, anti-HBc autoantibody is 25 persistent for the life of the animal. Several characteristics of HBeAg and HBcAg most likely explain these differences. The HBeAg is a secreted antigen, whereas the HBcAg is a cytosolic protein expressed in hepatocytes and only relatively small amounts of HBcAg leak out of the liver. Therefore, the presence of the autoantigen in the serum is contemplated to clear the autoantibody via immune complex formation and secondly via the constant exposure of HBeAg-specific T cells to soluble HBeAg which has been shown to be immunosuppressive through Fas- 30

mediated apoptosis (Milich *et al.*, J Immunol, 160:2013-2021, 1998). However, in the WHcAg platform system immune tolerance is predominantly relevant at the B cell level, because the insert is a self-B cell epitope, whereas the Th cells are specific for the WHcAg carrier. The reversibility of autoantibody production is dependent upon whether the antigen within the  
5 inoculum is driving antibody production mediated by WHcAg-specific Th cells or whether at some point the endogenous self protein itself drives autoantibody production. This is contemplated to occur if the expanded autoreactive B cells become efficient APCs to present the entire self protein to self-specific Th cells to an extent capable of breaking Th cell tolerance.

10                   **3. Alzheimer's Disease**

Cleavage of amyloid precursor protein (APP) yields amyloid- $\beta$ -peptide (A $\beta$ ) a 40-42 amino acid fragment which is present in abundance in the plaques or brain lesions found in Alzheimer's patients. Transgenic mice expressing human A $\beta$  serve as an animal model for Alzheimer's disease. Recently it was reported that antibody production to A $\beta$  in these transgenic  
15 mice inhibited plaque formation in the brains of the mice (Morgan *et al.*, Nature, 408:982-985, 2000). However, the A $\beta$  peptide was not very immunogenic and required many immunizations to induce rather low levels of antibody. The murine studies prompted several clinical trials, which were halted due to a number of cases of aseptic meningoencephalitis (Smith *et al.*, Lancet, 359:1864-1865, 2002). Two recent studies have more carefully mapped the specificity of the  
20 therapeutic anti-A $\beta$  antibodies and showed that the beneficial effects in mice arise from antibodies selectively directed against residues 4-10 of A $\beta$ <sub>42</sub>, which do not in themselves elicit an inflammatory response (Hock *et al.*, Nat Med, 8:1270-1275, 2002; and McLaurin *et al.*, Nat Med, 8:1263-1269, 2002). The A $\beta$ <sub>4-10</sub> epitope represents a B cell epitope possessing no predicted T cell sites. In contrast, use of the full-length A $\beta$ <sub>42</sub> is contemplated to have elicited an  
25 inflammatory T cell response. Furthermore, it was observed that the induction of IgG<sub>2b</sub> antibodies to A $\beta$ <sub>4-10</sub> had an optimal therapeutic effect. These recent studies have revived interest in an Alzheimer's disease vaccine. The WHcAg platform is well suited for this purpose because it has the potential to raise very high titer antibodies to the small A $\beta$ <sub>4-10</sub> epitope, without activating A $\beta$ -specific T cells. The WHcAg platform elicits a spectrum of IgG isotypes,  
30 (predominantly IgG<sub>2b</sub> isotype), although the platform and/or formulation are manipulable to

focus antibody production to a particular IgG isotype. The A $\beta$ <sub>4-10</sub> sequence and/or tandem repeats are inserted into the WHcAg platform at various positions inside and outside the loop, in combination with different C-termini. The platform(s) which is most immunogenic or otherwise advantageous (e.g., IgG isotype induction profile), is assessed in vaccination experiments in the 5 appropriate transgenic mouse model of Alzheimer's disease.

### C. Allergic Disorders

Simplistically, allergy occurs when exposure to an allergen (e.g., pollen) elicits an antibody of the IgE class, as opposed to an antibody of the IgG class. IgE antibody binds to a 10 particular cell type (Mast cell) and to the allergen, yielding an allergen-IgE complex on the surface of the Mast cell, which activates the Mast cell to release effector molecules such as histamine thereby mediating the symptoms of an allergic response. One allergy treatment termed desensitization involves injecting many doses of the allergen over long periods to bias the antibody response towards IgG rather than IgE production. The WHcAg technology is 15 contemplated to be useful in this application because WHcAg elicits strong IgG responses, but not IgE responses. B cell epitopes derived from known allergens are inserted into the WHcAg platform and used to immunize/desensitize allergic patients. Only one or two injections are contemplated to be necessary, in contrast to the numerous injections used in typical desensitization therapy. A number of linear peptidic B cell epitopes have been mapped for 20 common allergens including: peanut allergen (Ara h 3; Rabjohn *et al.*, Int Arch Allergy Immunol, 128:15-23, 2002); latex allergen hevein (Her b 5; Beezhold *et al.*, J Allergy Clin Immunol, 107:1069-1076, 2001); brown shrimp allergen (Pen a 1; Reese *et al.*, J Chromatogr B Biomed Sci Appl, 756:157-163, 2001); and the major grass pollen allergen (Phl p 1; Suphio *et al.*, FEBS Lett, 502:46-52, 2001). Often allergen-derived peptides lack IgE binding capacity, yet 25 anti-peptide IgG antibodies react with the native allergen and inhibiting IgE from binding to the native allergen (Focke *et al.*, FASEB J, 15:2042-2044, 2001). This is contemplated to occur via anti-peptide antibodies sterically hindering IgE binding, as well as by allergen clearance by anti-peptide IgG antibodies before IgE synthesis can occur. Hybrid WHcAg particles containing selected allergen-specific B cell epitopes are produced and selected for high titer IgG anti- 30 allergen production, which is examined for the capacity to inhibit patient IgE binding to mast or basophil cells.

## VII. Enhanced Particle Assembly Via Addition or Insertion of Acidic Amino Acids

### A. Hepadna Virus Core Antigens

During development of the present invention, the presence of a number of highly basic  
5 amino acids (especially K, R, H) in a candidate insert epitope was found to correlate negatively  
with the assembly of hybrid WHcAg particles and hybrid HBcAg particles (PCT/US01/25625;  
and Karpenko, *et. al.*, Amino Acids, 18:329-337, 2000). As shown in Table 17 and 18, the  
isoelectric point (pI) of epitope sequences inserted into the loop region, effects assembly of  
hybrid WHcAg and hybrid HBcAg particles. Previously, three parameters of the epitope insert  
10 that prevented self-assembly of hybrid HBcAg particles were identified: i) high epitope  
hydrophobicity; ii) large epitope volume; and iii) a high  $\beta$ -strand index.

The pI of the wild type WHcAg loop (76-82) region is approximately 6.14 and that of the  
wild type HBcAg loop approximately 4.12. Because the wild type WHcAg and HBcAg 76-82  
loop regions are acidic, the inventors predicted that epitope inserts more positively charged than  
15 the wild type sequence may have adverse effects on dimer formation (*e.g.*, particle subunit) and  
secondly, particle assembly (*e.g.*, core). Theoretically, excess positive charge in the loop may  
result in a repulsive force between the monomers and negatively effect dimerization or the  
efficient assembly of dimer subunits. However, an understanding of the mechanism(s) is not  
necessary in order to make and use the present invention.

20 Thus, several epitopes with pI's of seven or greater, which did not permit assembly of  
hybrid WHcAg or HBcAg particles, were either modified to contain the acidic amino acid  
glutamic acid or were bracketed by glutamic acid residues (*e.g.*, linker sequences). The effect of  
addition of glutamic acid substitutions or linker sequences to the insert sequence was then tested  
for the ability to rescue hybrid particle assembly. As shown in Table 18, in all cases addition of  
25 glutamic acid linker sequences rescued particle assembly on both the WHcAg and HBcAg  
platforms. Substitution of a non-acidic amino acid within the heterologous insert (at a  
nonessential position) with a glutamic acid residue also rescued hybrid particle assembly.  
Surprisingly even placement of negatively charged amino acids at a distance from the positively  
charged residues in the insert sequence resulted in hybrid particle assembly.

30 Additionally, the effect of addition of other amino acid residues on hybrid particle  
assembly was examined. A single basic (*e.g.*, pI = 8.74) epitope sequence was selected and

multiple amino acid linkers were tested. Interestingly, only peptidic linkers that significantly lowered the insert pI (*i.e.*, glutamic acid and aspartic acid) permitted WHcAg hybrid particle assembly. Thus, assembly of particles containing inserts with pIs of greater than seven are rescued by the addition of acidic acid substitutions or linker sequences. Therefore, when 5 possible a negatively-charged epitope should be selected. However, when this is not possible a positively-charged epitope is modified to include acidic amino acid substitutions and/or linker sequences, in order to obtain hybrid particles which assemble efficiently.

#### B. Other Self-Assembling Virus-Like-Particles (VLP)

Additional self-assembling virus-like-particles (VLP) are finding use as vaccine carrier platforms. A major, universal problem of VLP assembly has been the destabilizing effects of adding or inserting foreign peptidic sequences (PCT/US01/25625, Jegerlehner *et al.*, Vaccine, 20:3104, 2002; Chackerian *et al.*, J. Clin. Invest., 108:415-423, 2001; and Casal *et al.*, Methods 19:174-186, 1999). Hybrid VLP stability has represented such a serious problem that users of the HBcAg platform technology (Jegerlehner *et al.*, *supra*, 2002) and the Papillomavirus 10 platform technology (Chackerian *et. al.*, *supra*, 2001) have opted to chemically conjugate foreign epitopes to wild type VLPs, instead of attempting to produce hybrid particles via recombinant means. In particular, Chackerian found that the ability of L1-self-peptide chimeras to assemble 15 into VLPs was highly unpredictable. Similarly, Jegerlehner found that the size and nature of epitopes that can be inserted into the immunodominant region of VLPs was restricted and that VLPs containing inserts longer than 20 amino acids often failed to assemble. Similar to the hepadnavirus core proteins, preferred insertion sites on many VLPs are within the immunodominant exposed loop structures which are accessible for antibody recognition and which may be less likely to compromise the structural integrity of the particle, as opposed to 20 insertions into  $\alpha$ -helical or  $\beta$ -sheet regions (Sadeyen *et al.*, Virology 309:32-40, 2003). Thus, the inventors contemplate that the insertion of positively-charged epitopes into the exposed loop 25 region of other types of hybrid cores, is also expected to negatively effect assembly of these cores. Consequently, the inventors propose using acidic amino acids to rescue assembly of other viral core particles containing positively-charged epitopes. In fact, rational insertion or substitution of acidic amino acid residues into either the heterologous antigen of interest or the 30 vaccine platform, is contemplated to be useful for the following exemplary list of viruses from

which VLPs are obtained: human papillomavirus type 16 (Varsani *et al.*, J. Virol., 77:8386-8393); human papillomavirus type 11 (Rose *et al.*, J Virol., 67:1936-1944, 1993); hamster polyomavirus (Gedvilaite *et al.*, Virol., 20:21-35, 2000); hepatitis B virus (Pumpens *et al.*, Intervirol., 45:24-32, 2002); yeast Tyl and Ty3 (Roth, Yeast, 16:785-795, 2000); human immunodeficiency virus (Wagner *et al.*, Intervirol., 39:93-103, 1996); hepatitis C virus (Baumert *et al.*, Gastroenterology, 117:1397-1407, 1999); bovine rotavirus strain C486 (Sabara *et al.*, J. Virol., 65:6994-6997, 1991); norwalk virus (Ball *et al.*, Gastroenterology, 117:40-48, 1999); human parvovirus B19 (Brown *et al.*, J. Virol., 65:2702-2706, 1991); herpes simplex virus (Thomsen *et al.*, J. Virol., 68:2442-2457, 1994); poliovirus (Urakawa *et al.*, J. Gen. Virol., 70:1453-1463, 1989); RNA bacteriophage (Brown *et al.*, Intervirol., 45:371-380, 2002); bluetongue virus (French *et al.*, J. Virol., 64:5695-5700, 1990); duck hepatitis B virus (Paoletti *et al.*, Vaccine, 20:370-376, 2001); simian immunodeficiency virus (Yamshchikov *et al.*, Virol., 214:50-58, 1995); rabbit hemorrhagic disease virus (Plana-Duran *et al.*, Arch. Virol., 141:1423-1436, 1996); hepatitis E virus (Nikura *et al.*, Virol., 293:273-280, 2002); simian-human immunodeficiency virus (Yao, Res. Initiat. Treat Action, 8:20-21, 2003) and bovine leukemia virus (Kakker *et al.*, Virol., 265:308-318, 1999).

## **EXPERIMENTAL**

The following examples are provided in order to demonstrate and further illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

5 In the experimental disclosure which follows, the following abbreviations apply: eq (equivalents); M (Molar);  $\mu$ M (micromolar); N (Normal); mol (moles); mmol (millimoles);  $\mu$ mol (micromoles); nmol (nanomoles); g (grams); mg (milligrams);  $\mu$ g (micrograms); ng (nanograms); l or L (liters); ml (milliliters);  $\mu$ l (microliters); cm (centimeters); mm (millimeters);  $\mu$ m (micrometers); nm (nanometers);  $^{\circ}$ C (degrees Centigrade); U (units), mU (milliunits); min. (minutes); sec. (seconds); % (percent); kb (kilobase); bp (base pair); PCR (polymerase chain reaction); WHV (woodchuck hepatitis virus); GHV (ground squirrel hepatitis virus); HBV (human hepatitis B virus); HBcAg (hepatitis B core antigen); WT (wild type); OS (oligosaccharide); PS (polysaccharide); Tg (transgenic); TCR (T cell receptor); Th (helper T cell); MHC (major histocompatibility complex); TNF (tumor necrosis factor); IFN (interferon); mAb (monoclonal antibody), mIg (membrane immunoglobulin); APC (antigen presenting cell); MO (macrophage); LN (lymph node); SN (supernatant); RS (restriction site); ELISA (enzyme linked immunosorbent assay); MOI (multiplicity of infection); 1<sup>o</sup> (primary); and 2<sup>o</sup> (secondary).

10

The following reagents find use in the methods and compositions of the present invention: TCR-Tg mice: 7/16-5 mice recognizing HBcAg<sub>129-140</sub>/A<sup>b</sup>; 11/4-12 mice recognizing HBcAg<sub>129-140</sub>/A<sup>b</sup> (Chen *et al.*, J. Virol. 74:7587-7599, 2000); and 8/6-10 mice recognizing HBcAg<sub>120-131</sub>/A<sup>s</sup>; HBV-Tg mice: HBcAg-Tg mice (Milich *et al.*, J. Immunol. 152:455-466, 1994); and HBeAg-Tg mice (Milich *et al.*, Proc. Natl. Acad. Sci. USA, 87:6599-6603, 1990); particle-reactive antibodies (polyclonal anti-WHcAg particles; mAb 2221, anti-WHcAg monomers; mAb 3120, anti-HBcAg particles; and mAb 3105, anti-HBcAg loop); insert-reactive antibodies (mAb 2A10, anti-*P. falciparum* NANP; mAb 2B608, anti-*P. falciparum* NVDP; mAb 14C2, anti-influenza M2e; mAb TP2, anti-CETP; and mAb 2F2, anti-*P. vivax* repeat); CD40L plasmid pDC406-mCD40L (ATCC No. 68872); tetanus toxin plasmid pMEC4 (Dr. Locht, Pasteur, Lille, France); 50mer or smaller peptides (Invitrogen); plasmids PET11d and BL21(DE3) (Stratagene); and pLEX and K12 K802 *E. coli* (Invitrogen).

25

30

### **Example 1**

#### **Immunization of Mice**

Groups of 3-5 female mice, approximately 6-8 weeks old of various strains (either bred at the Vaccine Research Institute of San Diego, San Diego, CA or obtained from Jackson Laboratories, Bar Harbor, ME) were immunized intraperitoneally for antibody assays and subcutaneously for T cell assays. Antigens were injected in saline, or absorbed in 0.1% (w/v) AlPO<sub>4</sub> suspension, or emulsified in IFA or the squalene water-in-oil adjuvant Montanide ISA 720 (Seppic, Paris) depending on the experiment. Mice were bled pre-immunization and at various times after primary and booster immunizations for anti-insert/PS and anti-WHcAg antibody determinations. A larger number of mice/group (at least 10) were used to perform mouse potency (dose) studies because at limiting antigen doses, less than 100% of mice produce antibody, and the limiting dose was typically defined as the dose at which 50% of the mice produce antibody.

15

### **EXAMPLE 2**

#### **Antibody Assays**

Anti-WHcAg or peptide antibodies were measured in pooled or individual, murine sera by indirect solid phase ELISA using solid phase WT WHcAg (50ng/well) or insert peptide (0.5 µg/well) and goat anti-mouse Ig (or IgG isotype-specific) antibodies were used as the secondary antibody. The ELISA was developed with a peroxidase-labelled, affinity-purified swine anti-goat Ig. The data were expressed as antibody titer representing the highest dilution yielding three times (3X) the optical density of the pre-immunization sera. Anti-PS antibodies were measured in an identical manner on solid phase purified PSs (10 µg/ml), except that PolySorp plates (Nunc, Roskilde, Denmark) were used to coat PS antigens. Fifty micrograms of pneumococcal cell wall polysaccharide (C-PS) per ml of sera were added to absorb any anti-C-PS antibodies.

### **EXAMPLE 3**

#### **T Cell Assays**

To measure T cell proliferation, groups of 3-5 mice were primed with either 10 µg of WT core, hybrid core or PS-core conjugates by hind footpad injection. Approximately, 7-10 days

after immunization, draining popliteal lymph node (LN) cells were harvested, and  $5 \times 10^5$  cells in 0.1 ml of Click's medium were cultured with 0.1 ml of medium containing WT core, hybrid core or PS-core conjugates, various synthetic peptides, or medium alone. Cells were cultured for 96 hr at 37° C in a humidified 5% CO<sub>2</sub> atmosphere, and during the final 16 hr, 1 µCi of <sup>3</sup>H-thymidine (<sup>3</sup>H-TdR; at 6.7 Ci/mmol, New England Nuclear, Boston, MA) was added to each well. The cells were then harvested onto filter strips for determination of <sup>3</sup>H-TdR incorporation. The data were expressed as counts per minute corrected for background proliferation in the absence of antigen (Δcpm). The T cell nature of the proliferation was confirmed by analyzing nylon-wool column-enriched T cells in selected experiments. To measure cytokine production, identical culture procedures were used with the exception that 24-72 hr supernatants were harvested and analyzed for the presence of cytokines (IL-2, IL-4, IFNγ) in standard ELISPOT assays.

#### EXAMPLE 4

##### *In Vivo Antibody Production in Response to WHcAg Immunization*

It was not obvious that the WHcAg and the HBcAg would behave similarly as antigens or as immunogens, because the WHcAg and the HBcAg are only approximately 67% conserved at the amino acid level. In addition, the HBcAg and the WHcAg migrate very differently in a 1% agarose gel (See, Figure 4). Furthermore, the WHcAg and the HBcAg do not significantly crossreact at the antibody (B cell) level (See, Figure 6) and are only partially crossreactive at the CD4<sup>+</sup> T helper cell level (See, Figures 7-10). Therefore, a number of studies were conducted to determine the immunogenicity of the WHcAg for evaluation of its potential as a vaccine carrier platform.

Briefly, eight H-2 congenic murine strains, differing only in MHC-haplotype, were immunized with equal doses of WHcAg or HBcAg (7.0 µg) emulsified in incomplete Freund's adjuvant (IFA). As shown in Figure 5, the WHcAg elicited higher levels of anti-core antibodies in 5 (B10.BR, B10.D1, B10.D2, B10.M and B10.RIII) of the 8 strains and equivalent anti-core antibodies in 3 strains (B10.PL, B10.S and B10) as compared to the HBcAg (6 weeks after administration of a single dose). This analysis also indicated that there are no genetic nonresponders to the WHcAg consistent with what has been previously reported for the HBcAg (Milich and McLachlan, Science, 234:1398-1401, 1986). However, the hierarchy of responder

H-2 haplotypes differs somewhat for the WHcAg as compared to the HBcAg due to the fact that the two proteins are only partially cross-reactive at the CD4<sup>+</sup> T cell level and each protein possesses a unique repertoire of CD4<sup>+</sup> T cell epitopes including several shared epitopes. Figure 6 depicts the extremely low level of antibody cross-reactivity between the HBcAg and the WHcAg. Crossreactivity between anti-WHc and anti-HBc antibodies ranged between 0 and 0.8%. Similarly, a panel of monoclonal antibodies (mAb) specific for the HBcAg was found to be totally non-crossreactive with the WHcAg when tested for binding to solid phase HBcAg and WHcAg by ELISA. The anti-HBcAg mAb panel included #3105, #3120 (Takashi *et al.*, J.Immunol, 130:2903-2911, 1983), C1-5 (Chemicon, Temecula, CA), C3-1, #440 and #442 (Boehringer Mannheim, Germany), and H40-C47.

Additionally, the ability of the WHcAg to act as a T cell-independent antigen was tested, as this property had been previously reported for the HBcAg (Milich and McLachlan, *supra*, 1986). For this purpose, wild-type and T cell deficient athymic (nu/nu) mice were immunized with 10 µg the particulate WHcAg or the nonparticulate WHeAg (a secreted form of the WHcAg that exists in serum as a monomer) in complete Freund's adjuvant. As shown in Table 8, the WHcAg was capable of eliciting anti-WHc antibodies in athymic mice in the absence of Th cells, whereas, the nonparticulate WHeAg was strictly a T cell-dependent antigen.

**Table 8. *In Vivo* Antibody Production to WHcAg and WHeAg in Athymic Mice**

Strain	Immunogen	Bleed (day)	Antibody Titers (1/dilution)	
			Anti-WHc	Anti-WHe
Balb/c (+/+)	WHcAg	10	163,840	10,240
		24	40 x 10 <sup>6</sup>	2.6 x 10 <sup>6</sup>
	WHeAg	10	20,480	20,480
		24	2.6 x 10 <sup>6</sup>	655,360
Balb/c (nu/nu)	WHcAg	10	10,240	640
		24	10,240	0
	WHeAg	10	0	0
		24	0	0

## EXAMPLE 5

### CD4<sup>+</sup> T cell Responses to the WHcAg

Importantly, a carrier platform must possess sufficient Th cell (CD4<sup>+</sup>) recognition sites to ensure that every MHC haplotype will be able to associate with at least one T cell site in order to avoid genetic (MHC-linked) nonresponsiveness. Figure 7 illustrates the CD4<sup>+</sup> T cell proliferative response to WHcAg and HBcAg upon immunization of Balb/c mice with WHcAg. The WHcAg was able to recall a proliferative response at a relatively low *in vitro* concentration of 0.12 ng/ml. Also note the low level of crossreactivity between the WHcAg and the HBcAg. Specifically, the HBcAg required an *in vitro* concentration of 80 ng/ml to recall a proliferative response from WHcAg-primed T cells which amounts to a 666-fold difference from the recall response observed for WHcAg. This result and additional studies indicate that the WHcAg-primed T cells in Balb/c mice (H-2<sup>d</sup>) recognize a site(s) on WHcAg which is not conserved on the HBcAg. Specifically, B10.D2 mice (H-2<sup>d</sup>) recognize the p60-80 sequence of WHcAg, which is not conserved on the HBcAg sequence (*See*, Figure 8, panel B). Similarly, the B10.M (H-2<sup>f</sup>) strain also recognizes a T cell epitope within p60-80 on WHcAg, and WHcAg-primed T cells from this strain are poorly crossreactive with HBcAg (*See*, Figure 8, panel C). Alternatively, if an WHcAg-specific T cell recognizes a site on WHcAg which is highly conserved between WHcAg and HBcAg, than the WHcAg and HBcAg epitopes will be crossreactive for that particular T cell. Such a circumstance occurs in the B10.S (H-2<sup>s</sup>) strain in which the dominant T cell recognition site is within p120-131, a region which is highly conserved on HBcAg. Therefore, in strains bearing the H-2<sup>s</sup> haplotype the WHcAg and the HBcAg are crossreactive at the T cell (CD4<sup>+</sup>) level as shown in Figure 8, panel A. Thus, the T cell crossreactivity of the WHcAg and the HBcAg has been shown herein to be variable and dependent upon the T cell site recognized (as dictated by MHC genotype).

Additionally, the fine specificity of T cell recognition of the WHcAg and the HBcAg in 8 different H-2 congenic strains representing 8 separate MHC genotypes was determined using panels of WHcAg- and HBcAg-derived synthetic peptides as antigens (*See*, Figures 9 and 10). In the first place, all 8 MHC genotypes responded to the WHcAg at the T cell level (no genetic nonresponders). Secondly, in general, most strains recognize totally distinct sets of T cell sites on WHcAg and HBcAg, and even when similar regions are recognized, WHcAg-primed T cells rarely crossreact with HBcAg-derived peptides and vice versa. The two exceptions are the H-2<sup>s</sup>

and the H-2<sup>b</sup> haplotypes, which predominantly recognize the 120-131 (H-2<sup>s</sup>) and 129-140 (H-2<sup>b</sup>) sites, both of which are highly conserved between the WHcAg and the HBcAg. However, even in H-2<sup>s</sup> and H-2<sup>b</sup>-bearing strains a number of additional non-crossreactive T cell sites are recognized by WHcAg or HBcAg-primed T cells. For example, the T cells of B10 (H-2<sup>b</sup>) mice primed with WHcAg recognize non-crossreactive T cell sites at residues 60-80, 80-90 and 105-125 in addition to the 129-140 T cell site.

Strikingly, in comparing T cell (CD4<sup>+</sup>) recognition of WHcAg versus HBcAg, WHcAg was found to be a more efficient T cell immunogen than HBcAg. First, in most murine strains the WHcAg appears to possess a greater number and more potent CD4<sup>+</sup> T cell epitopes. Second, comparing the amount of IFN $\gamma$  produced by WHcAg-primed T cells recalled with WHcAg, with the amount of IFN $\gamma$  produced by HBcAg-primed T cells recalled with HBcAg reveals that WHcAg elicits greater IFN $\gamma$  production in all but one strain (*i.e.*, B10.D1). Third, when the WHcAg is used as the immunogen the ratio of IFN $\gamma$  produced after *in vitro* recall with WHcAg as opposed to *in vitro* recall with HBcAg is always greater than 1 (ranges between 3.0 for the B10 and B10.S strains, and 32 for the B10.M strain). In contrast, when the HBcAg is used as the immunogen the ratio of IFN $\gamma$  production after *in vitro* recall with HBcAg as compared to recall with WHcAg is 1 or less in 5 of the 8 strains tested. A T cell response, which is recalled more efficiently by a heterologous antigen than the priming antigen is referred to as a heteroclitic T cell response. The ability of the WHcAg to elicit a heteroclitic T cell response from HBcAg-primed T cells in 3 strains (*i.e.*, B10.D2, B10.PL and B10.RIII) indicates that the WHcAg is processed and/or presented by antigen presenting cells (APCs) more efficiently than the HBcAg.

#### EXAMPLE 6

##### **Effect of WHcAg Use as a Vaccine Platform on the Anti-HBc Diagnostic Assay**

One of the advantages to using WHcAg as a vaccine platform is because unlike HBcAg, WHcAg is not expected to elicit anti-HBc antibodies which could compromise the use of the commercial anti-HBc assay as a diagnostic marker of previous or current HBV infection. To directly examine this issue murine anti-HBc, anti-WHc and antisera to the HBcAg-based malaria vaccine candidate V12.PF3.1 and several WHcAg-based malaria-core particles were tested in a commercial anti-HBc assay (Sorin, Italy). This assay is an inhibition assay and positivity is measured by % inhibition. As shown in Figure 11, anti-HBc demonstrated 100% inhibition and

antisera to the V12-PF3.1 malaria vaccine candidate demonstrated 50% inhibition, respectively. The anti-V12-PF3.1 antisera only inhibited 50% because the malaria insert in the loop disrupts one of only two dominant endogenous B cell epitopes on HBcAg (Schodel *et al.*, J Virol, 66:106-114, 1992). In contrast, murine antisera to native WHcAg showed only low level 5 inhibition (16%), as did antisera to one of the WHcAg-based hybrid-core particles (150-3RC-M74), while antisera to a second WHcAg-based hybrid particle (HyW-M78) exhibited no inhibition. Therefore the use of WHcAg as a vaccine platform circumvents the problem of interference with the commercial anti-HBc assay.

10

#### EXAMPLE 7

##### WHcAg Effectiveness as a Vaccine Platform in HBV Chronic Carriers

Another problem with the use of HBcAg as a vaccine platform is the issue of immune tolerance that exists to HBcAg in chronic carriers of the HBV. The HBV is endemic in many parts of the world with an estimated 300-400 million HBV chronic carriers worldwide. The 15 HBcAg-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses in HBV chronic carriers are severely depressed and usually undetectable (Ferrari *et al.*, J Immunol, 145:3442-3449, 1990). As the WHV is not a human pathogen and because CD4<sup>+</sup> T cell recognition of WHcAg and HBcAg is mostly non-crossreactive, a vigorous WHcAg-specific Th cell repertoire is contemplated to be available in HBV chronic carriers. To explore this issue, an HBV-Tg mouse model of HBV 20 chronic infection was used in the following studies. HBeAg-Tg mice produce the secreted form of the HBcAg and HBeAg-Tg mice on a (B10.S x Balb/c)<sub>F1</sub> background are tolerant to the HBcAg at the Th cell level, mimicking the immune status of HBV chronic carriers (Milich *et al.*, Proc. Natl. Acad. Sci. USA, 87:6599-6603, 1990).

Wild-type (+/+) mice and HBeAg-Tg mice were immunized with either HBcAg, a 25 HBcAg-based malaria vaccine candidate (HBc-M, V12.PF3.1) or a WHcAg-based malaria vaccine candidate (WHc-M, HyW-M78). Both vaccine candidates possess the same malaria CS-derived B cell epitope (*i.e.*, NANPNVDP(NANP)<sub>3</sub>, set forth as SEQ ID NO:75). The HBc-M vaccine candidate also possesses a heterologous malaria-specific Th cell epitope referred to as the malaria universal T cell site (Calvo-Calle *et al.*, J Immunol, 159:1362-1373, 1997). As 30 shown in Figure 12 panel A, HBeAg-Tg mice are immune tolerant to the HBcAg and produce no anti-HBc antibody, whereas, the control (+/+) mice produce anti-HBc at weeks 2, 4 and 8 post

immunization. Likewise, immunization with the HBc-based V12.PF3.1 vaccine candidate also elicits significantly less anti-HBc antibody production and less anti-NANP antibody production in HBeAg-Tg mice as compared to the control (+/+) mice (Figure 12, panels B and D). Therefore, immune tolerance to the HBcAg was shown herein to adversely affect the ability of  
5 the HBcAg to perform as a vaccine platform for a malaria-specific B cell epitope. The low level of anti-core and anti-NANP antibodies that are produced may be due to the function of the heterologous universal T cell site or a novel Th cell epitope(s) created at the junction between the HBcAg and the inserted sequence. The (NANP)<sub>3</sub> sequence (SEQ ID NO:68) is not a T cell epitope in (B10.S x Balb/c)<sub>F1</sub> mice. Importantly, the HBeAg-Tg mice demonstrated no  
10 diminished ability to produce anti-WHc or anti-NANP antibodies when the WHcAg-based malaria vaccine candidate (HyW-M78) was used (Figure 12, panels C and E). Thus, the negative effects of immune tolerance to the HBcAg were circumvented by using the WHcAg as a vaccine platform for a malaria B cell epitope.

In additional studies, the HBcAg-primed T cells of (B10.S x Balb/c)<sub>F1</sub> mice were found  
15 to predominantly recognize the p120-140 sequence. The p120-140 sequence is highly conserved between WHcAg and HBcAg, and all three antigens recall *in vitro* IFN $\gamma$  production from HBcAg-primed T cells in +/+ mice (Figure 13, panel A). However, p120-140-specific T cells are tolerized in HBeAg-Tg mice (*i.e.*, no recall response with 120-140), which accounts for the poor *in vitro* recall responses elicited by both the HBcAg and the WHcAg compared to +/+ mice.  
20 (Figure 13, panel D). Similarly, the *in vitro* T cell responses to the HBcAg and the WHcAg are significantly reduced in HBeAg-Tg mice as compared to +/+ mice immunized with the HBcAg-based V12.PF3.1 vaccine candidate because 120-140-specific T cells are tolerized in HBeAg-Tg mice (Figure 13, panels B and E). In contrast, an advantage to using the WHcAg as a carrier platform is the presence of T cell epitopes within the WHcAg that are unique to the WHcAg and  
25 not present on the HBcAg, for example, residues 60-80 (W) and 80-95 (W) (Figure 13, panels C and F). Therefore, while the function of p120-140-specific T cells is lost in HBeAg-Tg mice immunized with the WHcAg-based vaccine (HyW-M78) due to the tolerizing effects of the presence of HBeAg, T cell recognition of the WHcAg-specific T cell epitopes (p60-80W and p80-95W) is identical in control (+/+) and HBeAg-Tg mice. The ability of the WHcAg to recall  
30 IFN $\gamma$  production in HBeAg-Tg mice is only marginally decreased as compared to the HBcAg due to the function of the p60-80 (W) and p80-95 (W) T cell sites, which are also sufficient to

promote high levels of anti-WHc and anti-NANP antibody production in HBeAg-Tg mice as shown in Figure 12. Thus, the WHcAg platform is contemplated to be significantly more effective in an HBV chronic carrier population than a vaccine based on the HBcAg platform.

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## EXAMPLE 8

### Versatility of the WHcAg Combinatorial Technology

Although the HBcAg has been used as a carrier platform, less than 50% of selected foreign sequences can be successfully inserted into HBcAg (See, Jegerlehner *et al.*, Vaccine, 20:3104, 2002; and International Application No. PCT/US01/25625, hereby incorporated by reference). This high failure rate is contemplated to be due to the destabilizing effects of inserting foreign sequences on particle assembly. To circumvent this problem, others have chosen to chemically link foreign epitopes to wild-type particles, as opposed to trying to incorporate the epitopes into the particles by recombinant methods (Jegerlehner *et al.*, *supra*, 2002, and Chackerian *et al.*, J Clin Invest, 108:415-423, 2001). In contrast, the current invention was developed to accommodate a greater variety of foreign epitope insertions without destabilizing particle assembly. Specifically, successful direct insertions of epitopes have been reported only for positions 77, 78, 81, 82 and the N- and C-termini of HBcAg (Pumpens and Grens, Intervirology, 44:98-114, 2001). On the other hand, using the WHcAg platform, in addition to positions 77, 78, 81, and 82 within the loop region and the N- and C-termini, a number of other internal insertion sites outside the loop region have been identified including positions 44, 71, 72, 73, 74, 75, 76, 83, 84, 85 and 92 (See, Figure 3). Importantly, during development of the present invention, three HIV epitopes, which could not be expressed and/or assembled using the HBcAg platform, were successfully expressed and assembled in the context of the WHcAg platform. Specifically, the WHcAg platform rescued the HIV4.1, HIV5.1, and HIV6.1 epitopes (See, Table 10), for which failures using HBcAg were previously reported (International Application No. PCT/US01/25625). In short, a relatively large library of 17 competent insertion sites on the WHcAg platform have been identified during development of the present invention.

Importantly, this expansion of the number of positions available for insertion of foreign epitopes was made possible by the generation of a library of C-terminal modifications to the WHcAg which variably stabilize insertions at different positions. In fact, the C-terminal

modifications of the WHcAg described herein comprise a very useful second library of 21 C-terminal modifications. Table 1 lists the sequences of the various modified C-termini. The C-terminal modifications were designed to eliminate RNA/DNA binding motifs, eliminate/substitute prolines, replace the last five C-terminal amino acids and to eliminate or 5 conserve non-homologous regions between HBcAg and WHcAg. Wild type or full length WHcAg binds significant amounts of bacterial RNA/DNA, which is undesirable for a vaccine platform. During development of the present invention, RNA/DNA binding to the C-terminally modified WHcAg particles has largely been eliminated. In contrast, only three different HBcAg C-termini have been previously described: full length; truncated at residue 149, and truncated 10 plus an added cysteine at position 150.

The combined libraries of insertion sites and modified C-termini accumulated for the WHcAg have permitted the successful insertion of 22 of 24 attempted sequences (See, Table 9). Additionally, the sequence of the inserted epitope has been found to play a role in determining whether a given sequence can be inserted at a given position in the context of a given C-terminus. The sequence of selected inserts is provided in Table 10. Therefore, three variables 15 must be considered in designing a WHcAg-hybrid particle: the insert position; the C-terminal sequence; and the epitope sequence. For this reason, a rapid screening method has been developed to examine efficacy of expression and assembly of hybrid-core particles at the early bacterial lysate step. This method makes feasible a combinatorial approach involving shuffling 20 of the insert position, and the C-terminal modification for each epitope of interest. As shown in Table 11, a strong correlation between the relative lysate assembly scores and the ability to purify hybrid core particles in high yield has been observed.

**Table 9. Summary of Insert Sites, Model Epitopes, and C-Termini  
Successfully Tested on the WHcAg Platform**

List of Insert Sites	List of Epitopes <sup>1</sup>	List of C-Termini
44-45	M	FL(188)
71-72	MV	150C
72-73	IM2	150R
73-74	IM2(-)	150-2RC
74-75	FV-1	150-3RC
75-76	FV-2	150-4RC
77-78	HV-1	150-3KC
78-79	HV-2	150-3AC
81-82	HV-3	WT-R
82-83	HV-4	WT-R1
83-84	HV 4.1	WT-R2
84-85	HV 5.1	WT-R3
85-86	HV 6.1	C-long
92-93	CETP	C-long(M1)
C-terminal	SEB	C-long(M2)
N-terminal	AZ	C-long(M3)
	HCV-6	HyW
	HCV-10	HyW-1
	HCV-17	HyW-2
	HCV-18	HyW-3
	HCV-24	HyW-4
	EGFR VIII	HyW-5
	OMP-1	
	OMP-2	

Abbreviations include: M, malarial CS repeat - *P. falciparum*; MV, malarial CS repeat type I - *P. vivax*; IM2, influenza A M2e extracellular domain; IM2(-), mutant influenza A M2e domain lacking two cysteine residues; FV, feline immunodeficiency virus-1 gp41, HV, human

immunodeficiency virus gp120; CETP, cholesteryl ester transfer protein; SEB, staphylococcus enterotoxin B; AZ,  $\beta$ -amyloid; HCV, hepatitis C virus; EGFR VIII, epidermal growth factor receptor mutant VIII; and OMP, outer membrane protein. To date 22 out of 24 epitopes tested were accommodated by the WHcAg vaccine platform (92% success rate). This is in contrast to the less than 50% success rate seen when using the HBcAg platform (wild type, I<sup>149</sup> C-terminus and C<sup>150</sup> C-terminus) as determined through review of the literature and through development of the present invention.

**Table 10. Primary Amino Acid Sequences of the Various Model Epitopes**

Designation <sup>1</sup>	Sequence	Identifier
HV-1	GEIKNCFSNISTSIRGVQKEYAFF	SEQ ID NO:70
HV-2	LTSCNTSVITQACPKVSFEPPIHYC	SEQ ID NO:71
HV-3	PKVSFEPPIHYCAPAGFAILKCNN	SEQ ID NO:72
HV-4	THGIRPVVSTQLLNGSLAEEE	SEQ ID NO:73
MV	DRAAGQPAGDRADGQPAG	SEQ ID NO:74
M	NANPNVDPNANPNANPNANP	SEQ ID NO:75
IM2	SLLTEVETPIRNEWGCRCNDSSD	SEQ ID NO:76
IM2(-)	SLLTEVETPIRNEWGARANDSSD	SEQ ID NO:77
SEB	KKKVTAQELD	SEQ ID NO:78
CETP	FGFPEHLLVDFLQSL	SEQ ID NO:79
FV-1	FYEIIMDIEQNNVQGKQGLQKL	SEQ ID NO:80
FV-2	MELRKNGRQCGMSEKEEE	SEQ ID NO:81
EGFR VIII	LEEKKGNYVVTDH	SEQ ID NO:82
AZ-1	DAEFRHDSGYEV	SEQ ID NO:83
AZ-2	FRHDSGY	SEQ ID NO:84
HV 4.1	RIKQIGMPGGK	SEQ ID NO:85
HV 5.1	LLELDKWASL	SEQ ID NO:86
HV 6.1	EQELLELDKWASLW	SEQ ID NO:87
HCV-6	DTGFLAAL	SEQ ID NO:88
HCV-10	YCFTPSPV	SEQ ID NO:89
HCV-17	CFRKHPEA	SEQ ID NO:90
HCV-18	EATYSRCG	SEQ ID NO:91
HCV-24	HLHQNIVD	SEQ ID NO:92

10

<sup>1</sup>See footnote to Table 9 for a key to epitope abbreviations.

**Table 11. Positive Correlation Between Expression and Assembly Scores  
and Hybrid Particle Purification<sup>1</sup>**

Designation	Expression (anti-pWHc)	Assembly (anti-nWHc)	Accessability (anti-insert)	Particle Purification
150-3KC-M74	3	4	4	yes
150-3AC-M74	4	3	4	yes
c-long(M3)-M74	3	3	4	yes
150C-M75	4	4	5	yes
150C-M77	4	3	2	yes
150C-M78	5	4	3	yes
C-long-M78	3	3	4	yes
HyW-M78	3	4	4	yes
HyW-M92	2	3	3	yes
HyW-M(NH2)	2	3	3	yes
HyW-M(COOH)	3	4	2	yes
HyW-MV78	4	3	4	yes
HyW-CE74	3	3	3	yes
HyW-HV-4(74)	2	2	ND	yes
150C-HV-4(78)	3	2	ND	yes
HyW-IM2(-)78	4	4	3	yes
HyW2-K75	3	3	ND	yes
average	3.24	3.24	3.4	n/a
150C-IM2(74)	3	2	3	no
HyW-IM2(74)	2	2	3	no
WT-R-IM2(74)	3	2	2	no
HyW-K78	2	2	ND	no
HyW-M74-CD40L(470)	2	2	2	no
average	2.4	2.0	2.5	n/a

<sup>1</sup>The scores shown are relative and designate antibody binding normalized to wild type (WT) WHcAg or maximal (MAX) binding for the insert-specific mAbs: 5 = WT/MAX; 4 = 5X less than WT, 3 = 25X less than WT, and 2 = 125X less than WT. ND = not determined.

## 5

## EXAMPLE 9

### Effect of Insert Position and C-Terminus on Particle Assembly

The position of the inserted epitope within the WHcAg has been shown herein to affect the ability of the hybrid WHcAg core to assemble as a particle. For example, the (M) epitope in the context of either HyW or HyW2 C-termini permitted assembly in most positions tested with 10 the exception of positions 21, 91 and 96 (See, Table 12). Similarly, positions 75, 76, 77, 78, 81, 82, and 83 were permissive in the context of the 150-C C-terminus. Note that position 74 (bold-type) was not permissive in the context of the 150-C C-terminus, but this position is rescued in the context of HyW/HyW2 C-termini. Similarly, position 78 is not permissive for assembly in the context of the 188-C C-terminus, but is permissive in combination with HyW/HyW2 and 15 150-C. Thus, the position of the insert can affect assembly and non-permissive insert positions can be rescued through combination with an alternate C-terminus. This phenomenon was not unique to malaria inserts, as similar effects were observed with other heterologous sequences.

Table 12. Effect of Insert Position on Hybrid Particle Assembly<sup>1</sup>

C-terminus	Epitope	Satisfactory Assembly	Poor/Non-Assembly
HyW/HyW2	M	44, 73, 74, 75, 78, 84, 85, 92, N, C	21, 91, 96
150C	M	75, 76, 77, 78, 81, 82, 83	66, 74, 79, 80, 86
188	M	74	78
HyW/HyW2	CE	74	75, 78
HyW2	FV-1	75, 78	74
HyW2	FV-2	74, 75, 78	-
150C	FV-1	75, 78	74
150C	FV-2	74, 75, 78	-
HyW/HyW2	HV-4	74, 75	-
150C	HV-4	75, 78	74

<sup>1</sup>Numbers represent the amino acid position on WHcAg that precedes the inserted epitope. Assembly was assessed by ELISA using core assembly-dependent anti-Hc antibody. Bold numbers depict insert positions that can be rescued by altering the C-terminus.

5        A second variable influencing hybrid particle assembly is the C-terminus of the WHcAg protein (See, Table 13). For example, the (M) epitope inserted at position 74 results in hybrid core particle assembly in the context of ten different C-termini, however, five C-termini are non-permissive for assembly with (M) at position 74. The (M) epitope in position 78 appears less destabilizing since most C-termini are permissive including all five of the C-termini which were  
10      non-permissive for this epitope inserted at position 74. Therefore, non-permissive C-termini can be rescued by altering the insert position. Interestingly, the two non-permissive C-termini for (M) at 78 are both permissive for (M) at 74. This reciprocal relationship indicates that the mechanisms of destabilization of the (M) insert at positions 74 and 78 are different and can be stabilized by different C-terminal sequences. Furthermore, the HyW and HyW2 C-termini  
15      appear to be significantly more permissive for a variety of inserted epitopes and positions than is the 150-C C-terminus. A summary of the combinatorial technology is depicted in Table 14. Ten of the heterologous model epitopes that have been used are listed together with the combination of C-terminus and insert position which resulted in an optimal platform. Note that for these ten heterologous epitopes, seven different combinations of C-terminus plus insert position are  
20      represented. In short as determined during development of the present invention, no one universal WHcAg platform suffices for all heterologous epitopes, and thus a combinatorial approach is necessary for the widest possible application of the WHcAg vaccine platform technology.

25        During development of the present invention, various WHcAg C-termini (seven) were used in place of the wild type HBcAg C-terminus. Specifically, three epitopes were inserted into modified WHcAg and into modified HBcAg at five different positions. As shown in Table 15, in all but one instance, the model epitopes expressed as part of a hybrid HBcAg containing a WHcAg C-terminus were assembled as virus-like particles. Therefore, the C-terminal  
30      modifications developed for WHcAg are also useful in the context of the HBcAg N-terminus.

**Table 13. Effect of C-terminal Modification on Hybrid Particle Assembly<sup>1</sup>**

Epitope	Insert	Satisfactory Assembly	Poor/Non-Assembly
M	74	<b>188, 150R, 150-3RC, 150-4RC, 150-3KC,</b> 150-3AC, C-long(M3), HyW HyW1, HyW2	<b>150C, C-long, C-long(M1),</b> <b>C-long(M2), WT-R</b>
M	78	<b>150C, HyW, 150-2RC, 150-3RC, C-long, C-</b> <b>long(M1), C-long(M2), C-long(M3), WT-R</b>	<b>150R, 188</b>
CE	74	HyW	150C
HV-2	75	HyW2	150C
HV-3	74	HyW2	150C
HV-3	75	HyW2	150C
HV-4	74	HyW	150C
CD40L (470)	C	188	150C, 150R
IM2(-)	78	HyW	150C

<sup>1</sup>Numbers represent the amino acid position on WHcAg that precedes the inserted epitope. Assembly was assessed by ELISA using core assembly-dependent anti-Hc antibody. Bold numbers depict C-termini that can be rescued by altering the insert position.

5

**Table 14. Optimal C-Terminus and Insert Position Combinations for Model Epitopes<sup>1</sup>**

Epitope	C-Terminus	Insert Position
M	C-long	78
MV	HyW	78
CE	HyW	74
FV-1	HyW2	75
FV-2	150C	74
HV-2	HyW2	75
HV-3	HyW2	75
HV-4	150C	75
IM2	WT-R	74
IM2(-)	HyW	78

<sup>1</sup>The amino acid sequences of the WHcAg C-termini and of the model epitopes are provided in Tables 1 and 10, respectively. The optimal platform determination was based upon either the immunogenicity of the purified hybrid particles or upon their assembly score.

**Table 15. Comparison of the WHcAg (W) and HBcAg (H) Vaccine Platforms<sup>1</sup>**

Epitope	Position	C-Terminus	Platform	Assembly
<b>M</b>	<b>92</b>	<b>HyW</b>	<b>W</b>	+
			<b>H</b>	+
<b>M</b>	<b>78</b>	<b>HyW2</b>	<b>W</b>	+
			<b>H</b>	+
<b>M</b>	<b>83</b>	<b>150C</b>	<b>W</b>	+
			<b>H</b>	+
<b>M</b>	<b>78</b>	<b>C-long</b>	<b>W</b>	+
			<b>H</b>	+
<b>M</b>	<b>78</b>	<b>C-long(M3)</b>	<b>W</b>	+
			<b>H</b>	+
<b>M</b>	<b>78</b>	<b>150-3KC</b>	<b>W</b>	+
			<b>H</b>	+
<b>M</b>	<b>78</b>	<b>WT-R</b>	<b>W</b>	+
			<b>H</b>	+
<b>CETP</b>	<b>74</b>	<b>HyW2</b>	<b>W</b>	+
			<b>H</b>	-
<b>CETP</b>	<b>75</b>	<b>HyW2</b>	<b>W</b>	-
			<b>H</b>	-
<b>CETP</b>	<b>78</b>	<b>HyW2</b>	<b>W</b>	-
			<b>H</b>	-
<b>IM2(-)</b>	<b>78</b>	<b>HyW2</b>	<b>W</b>	+
			<b>H</b>	+
<b>IM2(-)</b>	<b>78</b>	<b>150C</b>	<b>W</b>	-
			<b>H</b>	+

<sup>1</sup>Bold type highlights differences observed between the WHcAg and HBcAg platforms.

## EXAMPLE 10

### Immunogenicity of Hybrid Particles

A number of hybrid core particles containing different epitopes inserted at different positions with varying C-termini have been produced and purified during the development of the present invention (See, Table 16). The *in vivo* humoral immune response to the inserted epitope, as well as the WHcAg carrier, was assessed for these particles. Figures 14 and 15 provide a summary of antibody production 8 weeks after a primary (20 µg), and 6 weeks after a secondary (10 µg) immunization with the various hybrid particles emulsified in IFA for both injections.

Although a hierarchy of immunogenicity was observed, most hybrid core particles were quite immunogenic both in terms of anti-insert and anti-core antibody production. The primary anti-insert IgG serum titers ranged from 1:5000 to  $15 \times 10^6$  and the secondary anti-insert IgG serum titers ranged from 1:25,000 to  $1:75 \times 10^6$ . These very high levels of anti-insert antibody production (particularly over such a wide variety of inserted epitopes) are unprecedented in the hybrid VLP literature, and thus the effectiveness of the WHcAg vaccine platform was not predictable.

**Table 16. Exemplary Purified Hybrid WHcAg Particles**

Particle	Yield (mg/L)
188-M74	30
150C-M74	2
150R-M74	16
150C-M77	12
150C-M78	18
150C-HV4(78)	2
HyW-HV4 (74)	6
150C-3RC-M74	15
150C-3KC-M74	30
150C-3AC-M74	25
150C-M75	26
C-long-M78	16
HyW-M78	32
HyW2-IM2(-)81	30
150C-IM2(-)82	35
HyW2-SEB75	20
C-long-M3-M74	18
HyW-M74	21
HyW-M(COOH)	31
HyW-M(NH2)	16
HyW2-M75	12
HyW-IM2(-)78	10
HyW-CE74	16
HyW-K(COOH)	28
HyW2-LK75	26
HyW-M92	21
HyW-MV78	38
HyW-MV78/MF(NH2)	35
HyW2-FV-1(75)	22
188-CD40L	2

## EXAMPLE 11

### Effect of Heterologous Insert Position on Immunogenicity

The immunogenicity of hybrid core particles composed of the same HyW-modified C-terminus and the same malaria repeat epitope was found to vary depending on where the epitope (M) was positioned (See, Figure 16). Particles with insertions in (position 78) or near (position 74) the loop were more immunogenic in terms of the anti-insert response, than were particles with inserts fused to the N-terminus. Moreover, insert placement at the C-terminus was poorly immunogenic both in terms of end-point serum titer and delayed onset of antibody production. This correlation was not true for anti-carrier antibody production, which was greater or equal for the N- and C-terminal locations of the (M) epitope, as compared to the internal insertions. Therefore, the position of the epitope did not alter the overall immunogenicity of the particle and the positional effects are due to greater surface exposure and/or optimal spacing of the heterologous epitopes in or near the loop region. The high anti-carrier responses to the N- and C-terminally fused epitopes were contemplated to be due to conservation of the native loop structure and the endogenous WHcAg B cell epitopes on these hybrid core particles.

## EXAMPLE 12

### Effect of C-Terminus on Immunogenicity

The immunogenicity of particles with the malaria (M) epitope inserted at position 74 but with varied C-termini were compared as shown in Figure 17. Particles with the native full length (188-M74) or with the 150-3RC C-terminus were more immunogenic in terms of serum titers of anti-NANP antibody as well as a quicker onset (week 2) as compared to the 150 3AC and HyW C-termini. The particle comprised of the 150R C-terminus, which lacks a cysteine, was weakly immunogenic. The 150R-M74-hybrid particle was the least stable *in vitro* (and most likely *in vivo*), explaining the poorer immunogenicity results. The *in vitro* stability of the various other hybrid core particles is expected to correlate with immunogenicity *in vivo*.

Additionally, a bivalent hybrid core particle was constructed, containing the *Plasmodium falciparum* CS repeat epitope at the N-terminus and the *P. vivax* CS repeat (type 1) epitope in the loop position 78, HyW-MV78/M (NH<sub>2</sub>). As shown in Figure 15, during the primary response antibodies were produced to both inserts, although the serum titers were rather low (1:5000) as compared to most single inserts. However, after boosting, high titer antibodies were produced to

both CS repeat sequences. Thus, the present invention provides bivalent hybrid core particles containing highly immunogenic epitopes at different positions on the same particle.

### EXAMPLE 13

#### 5      Effect of Genetics on Immunogenicity of a WHcAg-based Malaria Vaccine

Efforts to produce *P. falciparum* vaccine candidates based on the CS repeat sequences have been plagued by low immunogenicity and severe genetic restriction characterized by low responders in human clinical trials, and low or nonresponder murine MHC genotypes in mouse immunization studies. To address this issue, CS-derived CD4<sup>+</sup> T cell epitopes such as CS<sub>326-345</sub> 10 were included (Calvo-Calle *et al.*, J Immunol, 159:1362-1373, 1997), although murine strains differ in responsiveness to CS<sub>326-345</sub>. Therefore, for pathogen-specific B cell epitopes and in particular for malaria B cell epitopes, it is imperative that the carrier platform provide sufficient T cell helper function in the context of a wide variety of MHC haplotypes to eliminate genetic nonresponsiveness.

15      To directly examine the issue of MHC-linked restriction of the antibody response to a WHcAg-based vaccine, B10 H-2 congenic murine strains expressing eight different H-2 haplotypes were immunized with a 10 µg dose of a WHcAg-malaria vaccine candidate (HyW-M78) in IFA. Both primary (1<sup>o</sup>, 6 weeks) and secondary (2<sup>o</sup>) anti-WHc and anti-NANP serum antibody titers were determined as shown in Figure 18. First and importantly, all H-2 haplotypes 20 responded and produced both anti-WHc and anti-NANP antibodies after a primary immunization with HyW-M78 (no nonresponder H-2 haplotypes were identified). Secondly, all strains at all time points produced an equal or greater antibody response to the insert (anti-NANP) as compared to anti-WHc, with the exception of the secondary antibody responses of the B10.S strain. The lack of genetic nonresponders to this experimental WHcAg-based vaccine is 25 consistent with the absence of nonresponders to the WHcAg platform itself at the antibody (Figure 5) and T cell (Figures 9 and 10) levels as determined during development of the present invention.

## EXAMPLE 14

### Complexity of T Cell Recognition of Hybrid Particles

The insertion of a foreign B cell epitope is contemplated to at times itself either represent a novel T cell site or create a novel T cell site at the junction between the inserted sequence and the core sequence. Also, it is contemplated that an insertion at times disrupts an endogenous T cell site. Unexpectedly, inserting the NANPNVDP(NANP)<sub>3</sub> epitope (SEQ ID NO:75) into the WHcAg platform, has been observed to cause the loss and/or gain of novel WHcAg-specific T cell sites unrelated to interrupting an endogenous T cell site or creating a junctional T cell site, respectively. As shown in Figure 19, pairs of H-2 congenic mice were immunized with wild-type WHcAg or the HyW-M78 hybrid particle containing the malaria CS repeat epitope. T cell fine specificity was mapped using a panel of WHcAg-derived synthetic peptides as antigens.

Note that in B10.D2 mice, immunization with HyW-M78 caused a loss of one very strong T cell site (amino acids 90-105) and the gain of one very strong T cell site (amino acids 80-95) as compared to WHcAg immunization. The dominant T cell site at amino acids 60-80 was functional for both immunogens. The loss of the amino acid 90-105 site was not obvious because the insertion at amino acids 78-79 does not directly interrupt the amino acid 90-105 sequence. Similarly, the new T cell site at amino acids 80-95, was downstream of the inserted sequence.

Likewise, in the B10.PL strain, two new rather weak T cell sites were gained (amino acids 1-15 and amino acids 50-70) and a very strong T cell site (amino acids 15-35) was converted into an intermediate T cell site by the insertion of a malaria B cell epitope. In the B10.RIII strain three of the four T cell sites recognized on native WHcAg were either lost or weakened when the HyW-M78 hybrid particle was used as the immunogen. Lastly, the B10 strain was found to recognize the (NANP)<sub>n</sub> sequence as a T cell site, as well as a B cell epitope.

This insertion of a novel T cell site caused the loss of one T cell site (amino acids 105-125), and converted a very strong T cell site into a weak T cell site (amino acids 60-80). Thus, the variability of T cell recognition caused by the insertion of foreign sequences even at a distance from endogenous T cell sites emphasizes the necessity for a carrier platform to possess a multiplicity of T cell recognition sites relevant to any given MHC genotype. The WHcAg satisfies this requirement as demonstrated by the direct mapping of numerous T cell sites relevant for each of eight different MHC genotypes, and by the absence of nonresponder MHC

haplotypes corresponding to the HyW-M78 candidate malaria vaccine. Additionally, the recognition of the (NANP)<sub>n</sub> sequence as a T cell site by B10 mice after immunization with HyW-M78, indicates that the WHcAg platform serves as a vaccine carrier for heterologous T cell (CD4+) epitopes, as well as B cell epitopes.

5

#### EXAMPLE 15

##### Effects of Insert pI and Linker Addition on the Assembly of Hybrid WHcAg and Hybrid HBcAg Particles

During development of the present invention, the effect of insert isoelectric point (pI) on assembly of hybrid hepadna virus core particles was assessed. The predicted pI shown below was calculated using the MacVector software program version 6.5.3, (Oxford Molecular Group, plc). The use of other programs, such as ProtParam Tool and Compute pI/MW (available on the ExPASy proteomics server of the Swiss Institute of Bioinformatics), for predicting the pI of an insert peptide sequence was found to give slightly different pI values. However, as used herein, the predicted pI calculated using MacVector is considered to be equivalent to the predicted pI calculated using ProtParam Tool, Compute pI/MW and any analogous algorithms.

As shown in Table 17, positively charged inserts (e.g., pI equal to or greater than 7.0) appear to adversely effected assembly of hybrid WHcAg or HBcAg particles. However, using the methods and compositions described herein, the addition of acidic substitutions or linker residues was found to be useful for neutralizing the apparent destabilizing effect of positively-charged inserts (high pI) on particle assembly. As shown in Table 18, the addition of acidic residues rescued hybrid-core particle assembly on both the WHcAg and HBcAg vaccine platforms. Nonetheless, an understanding of the mechanism(s) is not necessary in order to make and use the present invention.

Additionally, the rescue of a model positively-charged insert was made possible through the use of either flanking glutamic acid residues (EE-insert-EE), or flanking aspartic acid residues (DD-insert-DD). In contrast, neither flanking nonpolar residues (L-insert-L and P-insert-P), nor flanking uncharged polar residues (QQ-insert-QQ, TT-insert-TT, and YY-insert-YY) were able to convert an assembly-incompetent, positively-charged insert into an assembly-competent insert.

**Table 17. Correlation Between Insert pI and Hybrid Particle Assembly<sup>1</sup>**

Epitope	Sequence	Identifier	pI	Particles
FMDV*	RYNRNAVPLRGDLQVLAQKVARTLF	SEQ ID NO:93	12.01	-
HIV4.1*	RIKQIGMPGGK	SEQ ID NO:85	11.30	-
<i>P. yoelii</i> *	TAVVHQLKRKH	SEQ ID NO:94	11.30	-
HIV10.1*	HLLQLTVWGIKQLQAR	SEQ ID NO:95	11.14	
IgE <sub>413-435</sub> *	GETYQSRVTHPHLPRALMRSTTK	SEQ ID NO:96	11.13	-
P450-1A2*	GRERRPRLSDRPQLPYLEA	SEQ ID NO:97	10.92	-
HV-1	GEIKNCFSNISTSIRGKVQKEYAFF	SEQ ID NO:70	9.41	-
HV-3	PKVSFEPPIHYCAPAGFAILKCNN	SEQ ID NO:72	8.68	-
SEB	KKKVTAQELD	SEQ ID NO:78	8.63	+/-
HV-2	LTSCNTSVITQACPKVSFEPPIHYC	SEQ ID NO:71	7.00	-
AZ2	FRHDSGY	SEQ ID NO:84	7.00	-
FV-2	MELRKNGRQCGMSEKEEE	SEQ ID NO:81	4.86	+
HV-4	THGIRPVVSTQLLLNGSLAEEE	SEQ ID NO:73	4.55	+
FV-1	FYEIMDIEQNNVQGKQGLQKL	SEQ ID NO:80	4.46	+
MV	DRAAGQPAGDRADGQPAG	SEQ ID NO:74	4.20	+
CETP	FGFPEHLLVDFLQLS	SEQ ID NO:79	4.11	+
AZ1	DAEFRHDSGYEV	SEQ ID NO:83	4.08	+
IM2(-)	SLLTEVETPIRNEWGARANDSSD	SEQ ID NO:77	3.95	+
M	NANPNVDPNANPNANPNANP	SEQ ID NO:75	3.43	+
MB	DPPPPNPNDPPPNNP	SEQ ID NO:98	3.22	+

<sup>1</sup> Asterisk denotes negative assembly on HBcAg as previously reported  
(PCT/US01/25625).

**Table 18. Effect of Acidic Amino Acid Addition on Assembly of Hybrid Particles**

Epitope	Sequence <sup>1</sup>	Identifier	pI	Particles
<b>WHcAg Platform</b>				
SEB	KKKVTAQELD	SEQ ID NO:78	8.63	+/-
SEB2E	<b>EE</b> KKKVTAQELDEE	SEQ ID NO:99	4.20	+
AZ2	FRHDSGY	SEQ ID NO:84	7.00	-
AZ2E	<b>EE</b> FRHDSGYEE	SEQ ID NO:100	4.02	+
HIV4.1	RIKQIGMPGGK	SEQ ID NO:85	11.3	-
HIV4.1E	<b>E</b> ERIKQIGMPGGKEE	SEQ ID NO:101	4.74	+
<b>HBcAg Platform</b>				
AZ2	FRHDSGY	SEQ ID NO:84	7.00	-
AZ2E	<b>EE</b> FRHDSGYEE	SEQ ID NO:100	4.02	+
HIV4.1*	RIKQIGMPGGK	SEQ ID NO:85	11.3	-
HIV4.1E	<b>E</b> ERIKQIGMPGGKEE	SEQ ID NO:101	4.74	+

<sup>1</sup>Acidic substitutions or linker additions are shown in bold, while asterisks denote negative assembly on HBcAg as previously reported (PCT/US01/25625).

5 All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be

10 unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology, genetics, or related fields are intended to be within the scope of the following claims.

## **CLAIMS**

We claim:

5        1. A composition comprising a heterologous antigen linked to the amino acid sequence set forth in SEQ ID NO:58, said amino acid sequence comprising a loop region and further comprising from 1 to 100 amino acids at the carboxy end of residue I<sup>149</sup>, and wherein said 1 to 100 amino acids does not consist of C<sup>150</sup> or the sequence set forth as SEQ ID NO:42.

10        2. The composition of Claim 1, wherein said 1 to 100 amino acids is chosen from R<sup>150</sup>, K<sup>150</sup>, A<sup>150</sup>, R<sup>150</sup>R<sup>151</sup>C<sup>152</sup>, and SEQ ID NOS:3-6, 43-56.

15        3. The composition of Claim 1, wherein said 1 to 100 amino acids is chosen from SEQ ID NOS:2, 7-20.

15        4. The composition of Claim 1, wherein said 1 to 100 amino acids is chosen from SEQ ID NOS:22-36.

20        5. The composition of Claim 1, wherein said amino acid sequence further comprises at least one immune enhancer sequence.

25        6. The composition of Claim 1, wherein said heterologous antigen is inserted at a position within said loop region.

25        7. The composition of Claim 6, wherein said position within said loop region is chosen from amino acid residues 77, 78, 81, and 82.

30        8. The composition of Claim 6, wherein said position within said loop region is at amino acid residue 76.

9. The composition of Claim 1, wherein said heterologous antigen is inserted at a position outside of said loop region.

10. The composition of Claim 9, wherein said position outside said loop region is  
5 chosen from amino acid residues 71, 72, 73, 74, 75, 83, 84, 85, 92, N-terminal and C-terminal.

11. The composition of Claim 9, wherein said position outside said loop region is at amino acid residue 44.

10 12. The composition of Claim 1, wherein said heterologous antigen is inserted at a position within said loop region, and in a position outside said loop region.

13. The composition of Claim 1, wherein said heterologous antigen is conjugated to said amino acid sequence.

15 14. The composition of Claim 1, wherein said heterologous antigen comprises at least one B cell epitope.

15 15. The composition of Claim 1, wherein said heterologous antigen comprises at least  
20 one T helper cell epitope.

16. The composition of Claim 1, further comprising human hepatitis B virus core antigen chosen from wild type human hepatitis B virus core antigen and modified human hepatitis B virus core antigen lacking a heterologous antigen.

25 17. A nucleic acid sequence encoding said heterologous antigen linked to the amino acid sequence set forth in SEQ ID NO:58, of Claim 1.

18. An expression vector comprising the nucleic acid sequence of Claim 17.

19. A composition comprising the amino acid sequence set forth in SEQ ID NO:58, said amino acid sequence comprising a loop region and further comprising from 1 to 100 amino acids at the carboxy end of residue I<sup>149</sup>, and wherein said 1 to 100 amino acids does not consist of C<sup>150</sup> or the sequence set forth as SEQ ID NO:42.

5

20. The composition of Claim 19, wherein said 1 to 100 amino acids is chosen from R<sup>150</sup>, K<sup>150</sup>, A<sup>150</sup>, R<sup>150</sup>R<sup>151</sup>C<sup>152</sup>, and SEQ ID NOS:3-6, 43-56.

21. A method, comprising:

10

- a) providing:
  - i) a mammalian subject; and
  - ii) a composition comprising one or more of a polypeptide comprising a heterologous antigen linked to the amino acid sequence set forth in SEQ ID NO:58, said amino acid sequence comprising a loop region and further comprising from 1 to 100 amino acids at the carboxy end of residue I<sup>149</sup>, and wherein said 1 to 100 amino acids does not consist of C<sup>150</sup> or the sequence set forth as SEQ ID NO:42, and an expression vector encoding said polypeptide; and
- b) administering said composition to said subject under conditions such that an immune response is generated.

15

22. The method of Claim 21, wherein said immune response comprises one or more of lymphocyte proliferative response, cytokine response and antibody response.

20

23. The method of Claim 22, wherein said antibody response comprises production of IgG antibodies.

24. The method of Claim 23, wherein said IgG antibodies comprise an autoantibody.

25. A method for producing an immunogenic composition, comprising:

- a) providing:
  - i) a heterologous antigen; and
  - ii) a human hepatitis B virus core antigen;
- 5 b) altering at least one of said heterologous antigen and said human hepatitis B virus core antigen, with a modification chosen from insertion of at least one acidic amino acid residue and substitution of at least one acidic amino acid residue; and
- c) inserting said heterologous antigen of step b within said human hepatitis B virus core antigen of step b, to produce a modified human hepatitis B virus core antigen;
- 10 d) expressing said modified human hepatitis B virus core antigen under conditions suitable for producing particles having a diameter of 25 to 35 nm.

26. The method of Claim 25, wherein in the absence of said altering, expression of said modified human hepatitis B virus core antigen yields 25 fold less particles than does expression of a wild type human hepatitis B virus core antigen.

27. The method of Claim 25, wherein said at least one acidic amino acid residue comprises at least one aspartic acid residue and at least one glutamic acid residue.

20

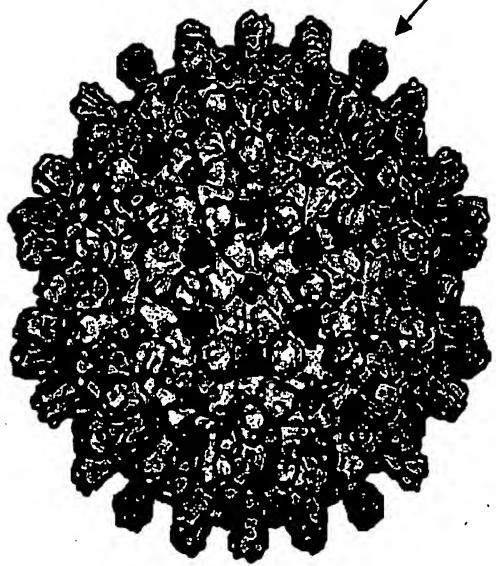
28. The method of Claim 25, wherein said insertion is in at least one position chosen from the N-terminus and the C-terminus of said heterologous antigen.

29. The method of Claim 25, wherein said substitution comprises a replacement of at least one non-acidic amino acid residue within said heterologous antigen, with said at least one acidic amino acid residue.

30. The method of Claim 25, wherein said altering produces a modified heterologous antigen with an isoelectric point in the range of 3.0 to 6.0.

30

**76-82**



**Fig. 1**

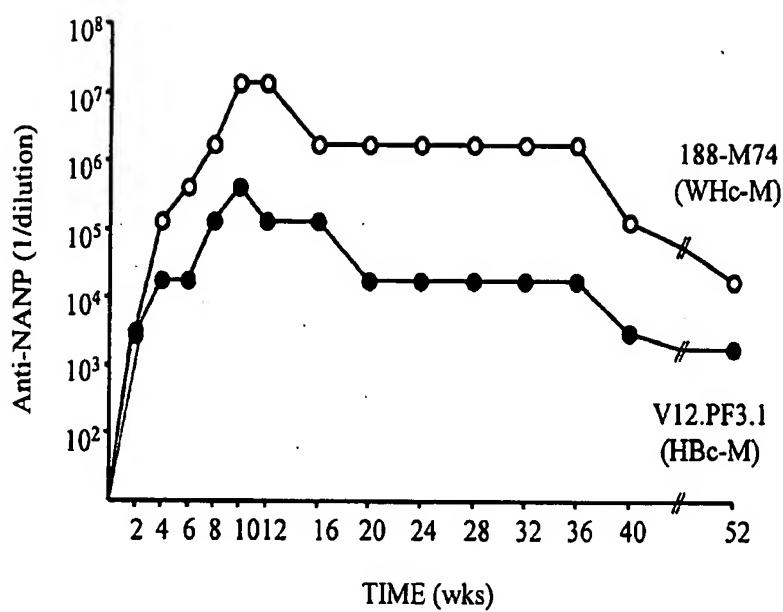
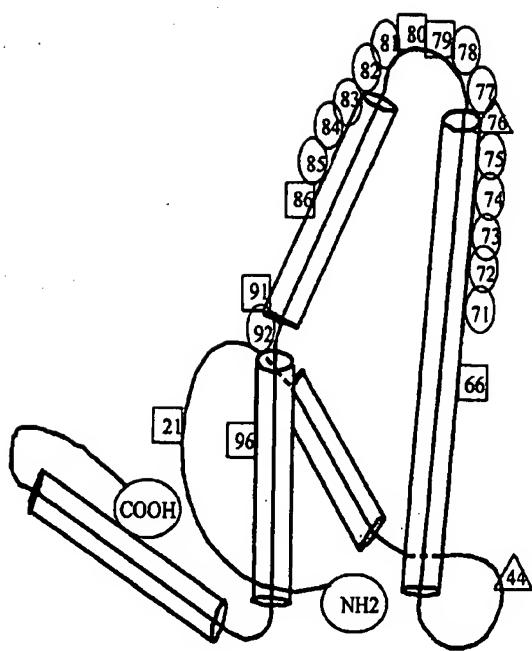


Fig. 2

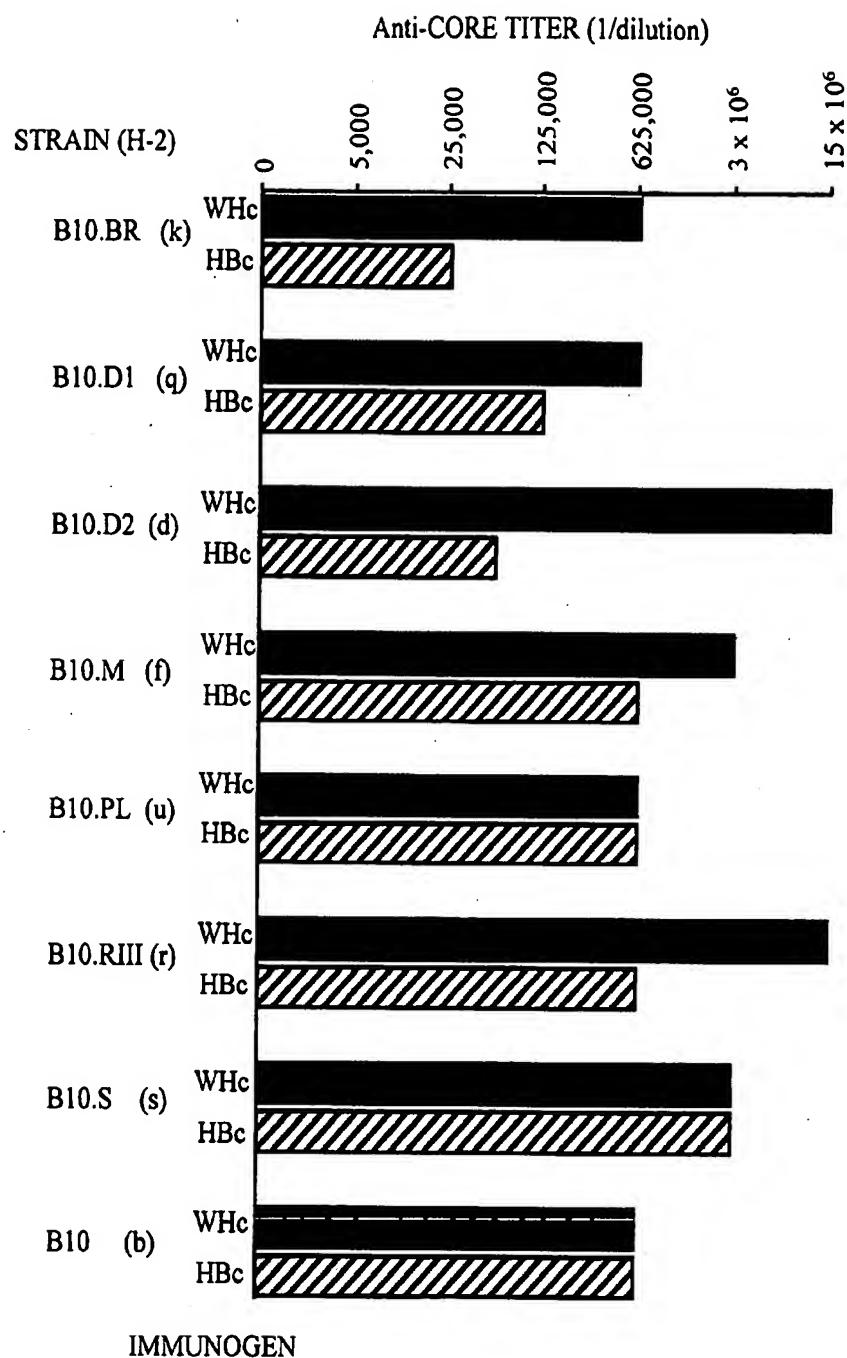


- tolerant
- △ intermediate
- intolerant

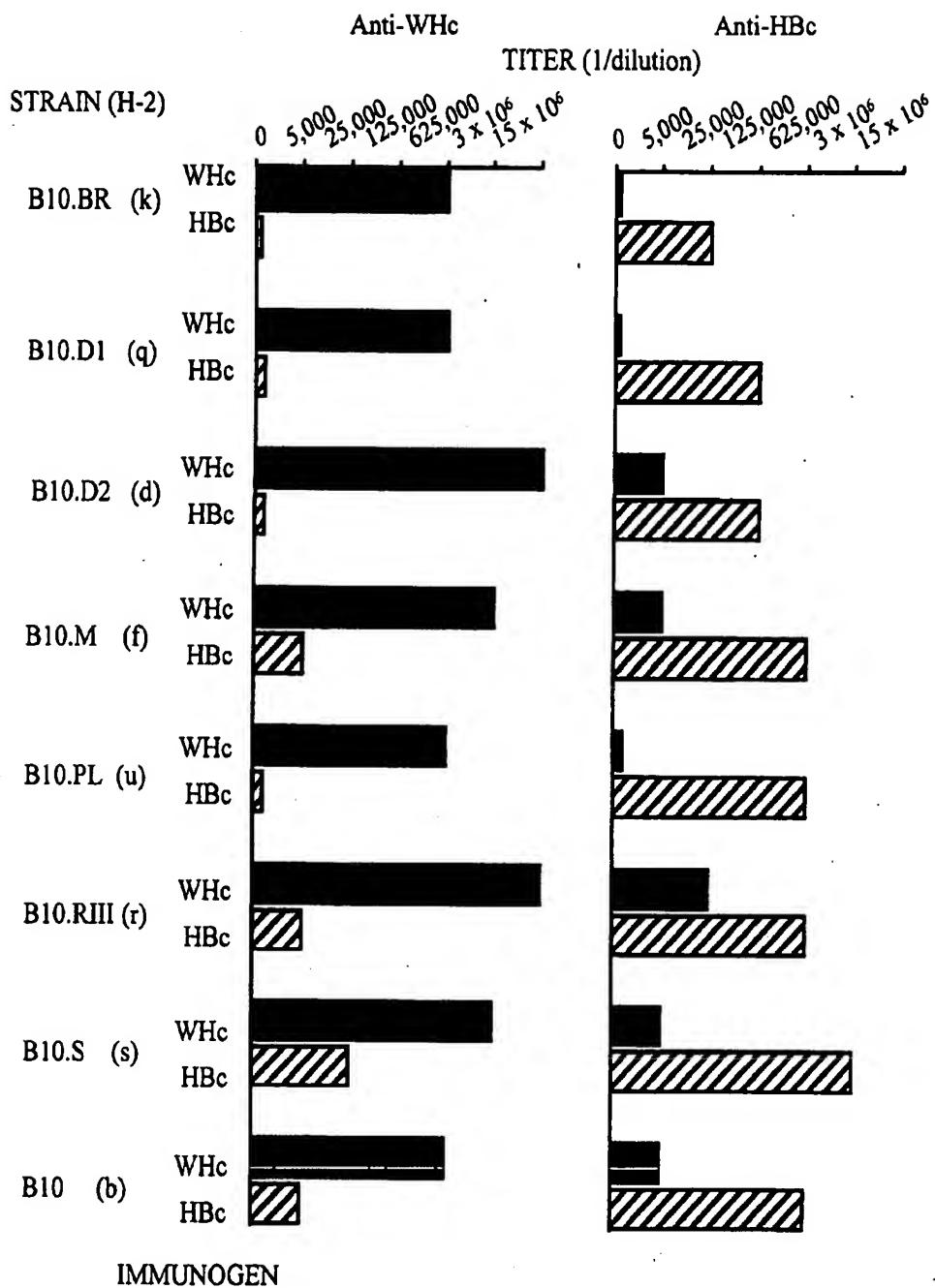
Fig. 3



**Fig. 4**



**Fig. 5**



**Fig. 6**

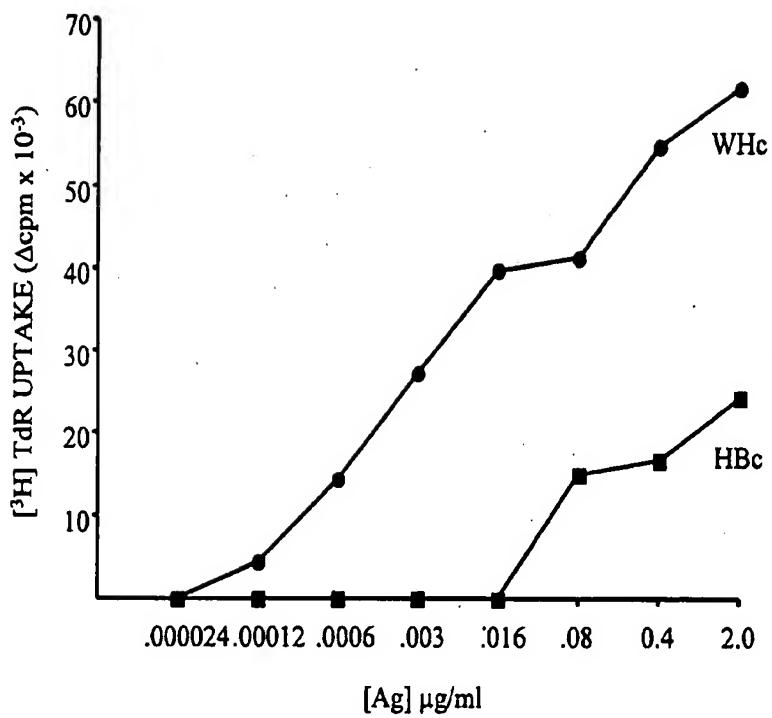


Fig. 7

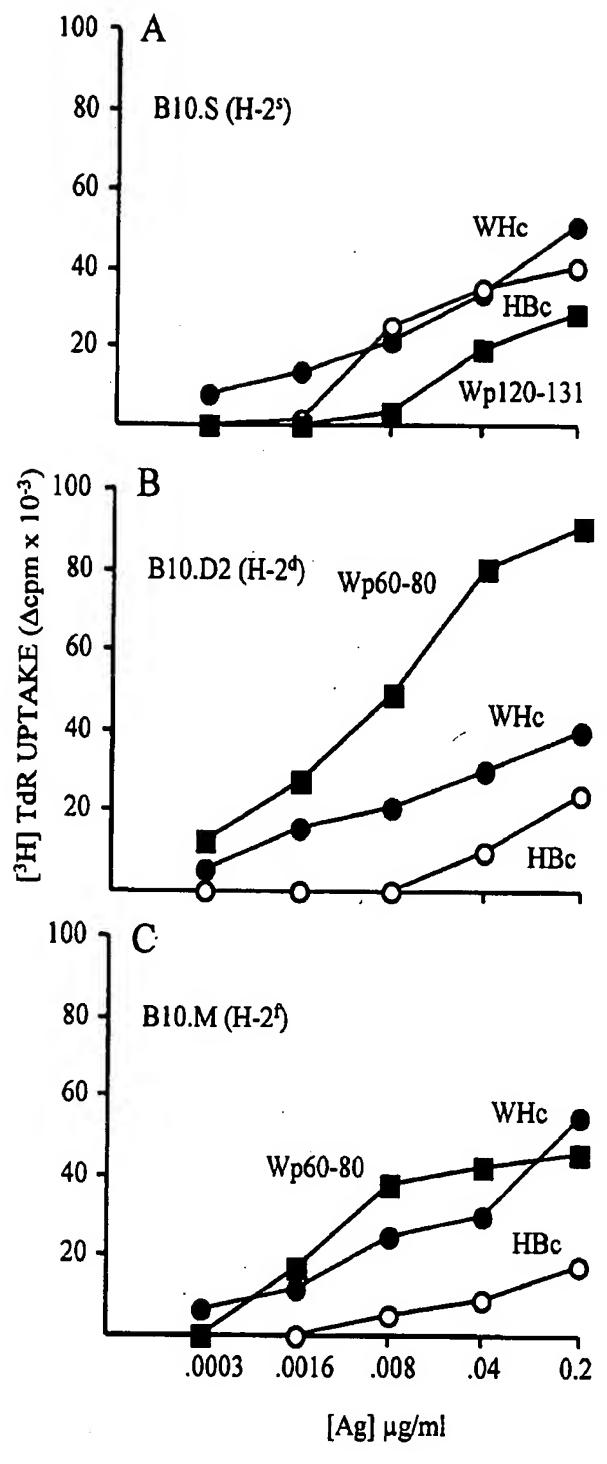


Fig. 8

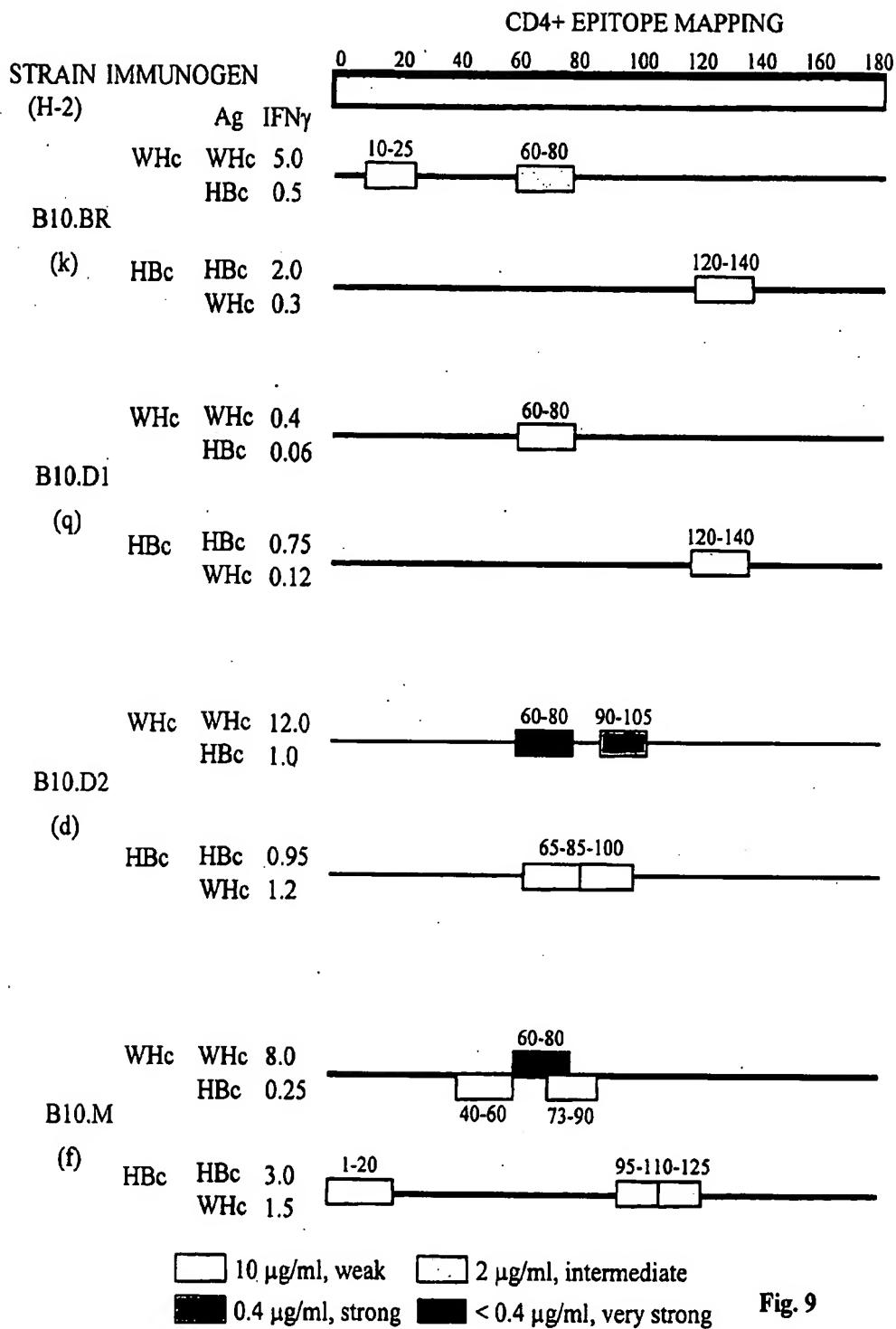
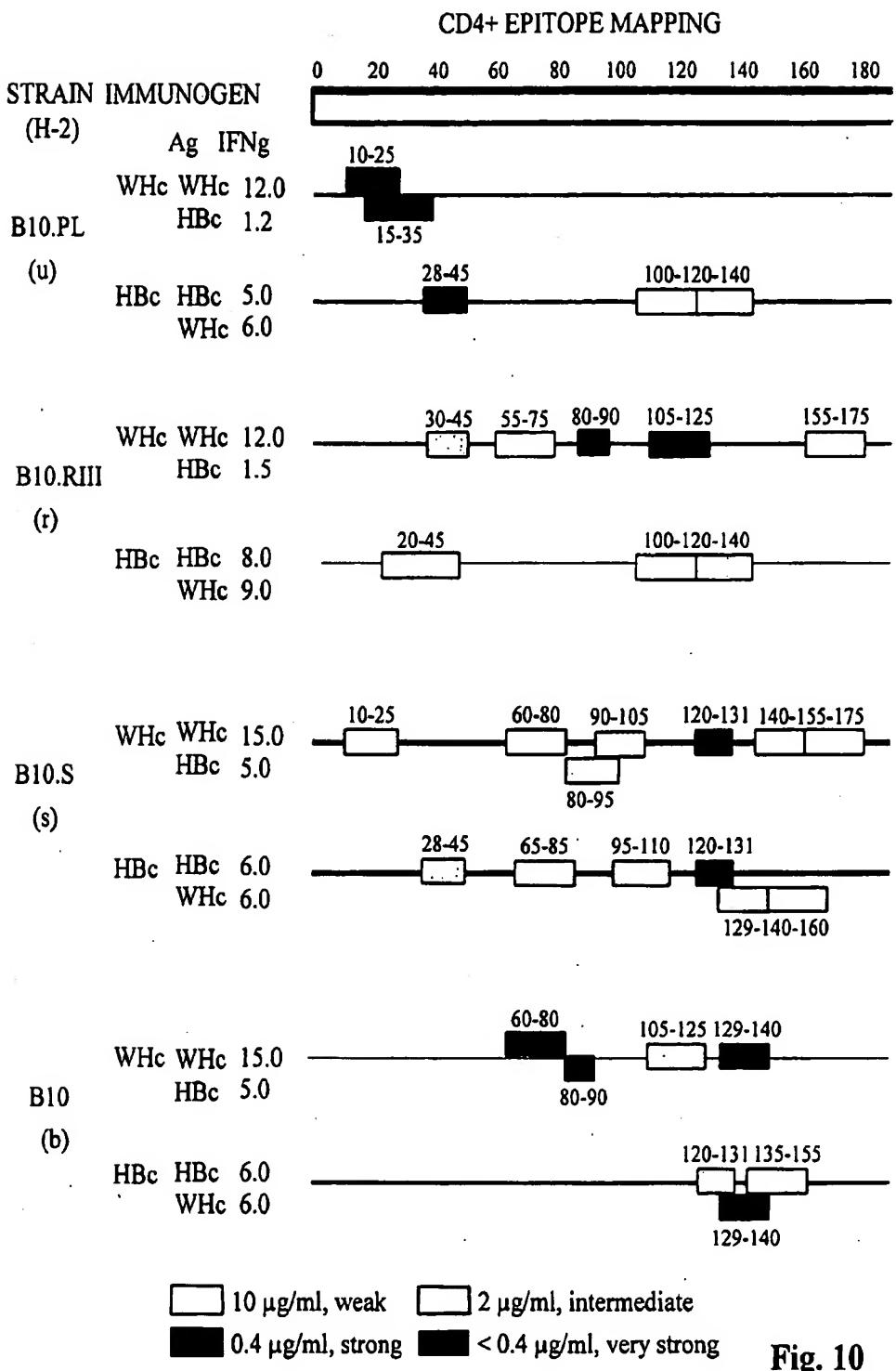


Fig. 9



**Fig. 10**

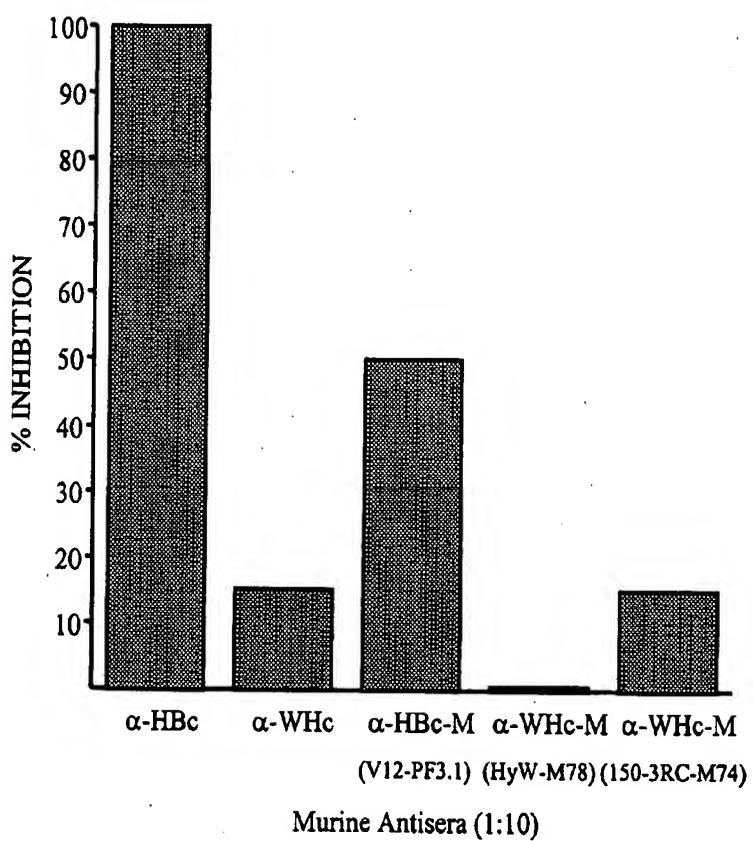


Fig. 11

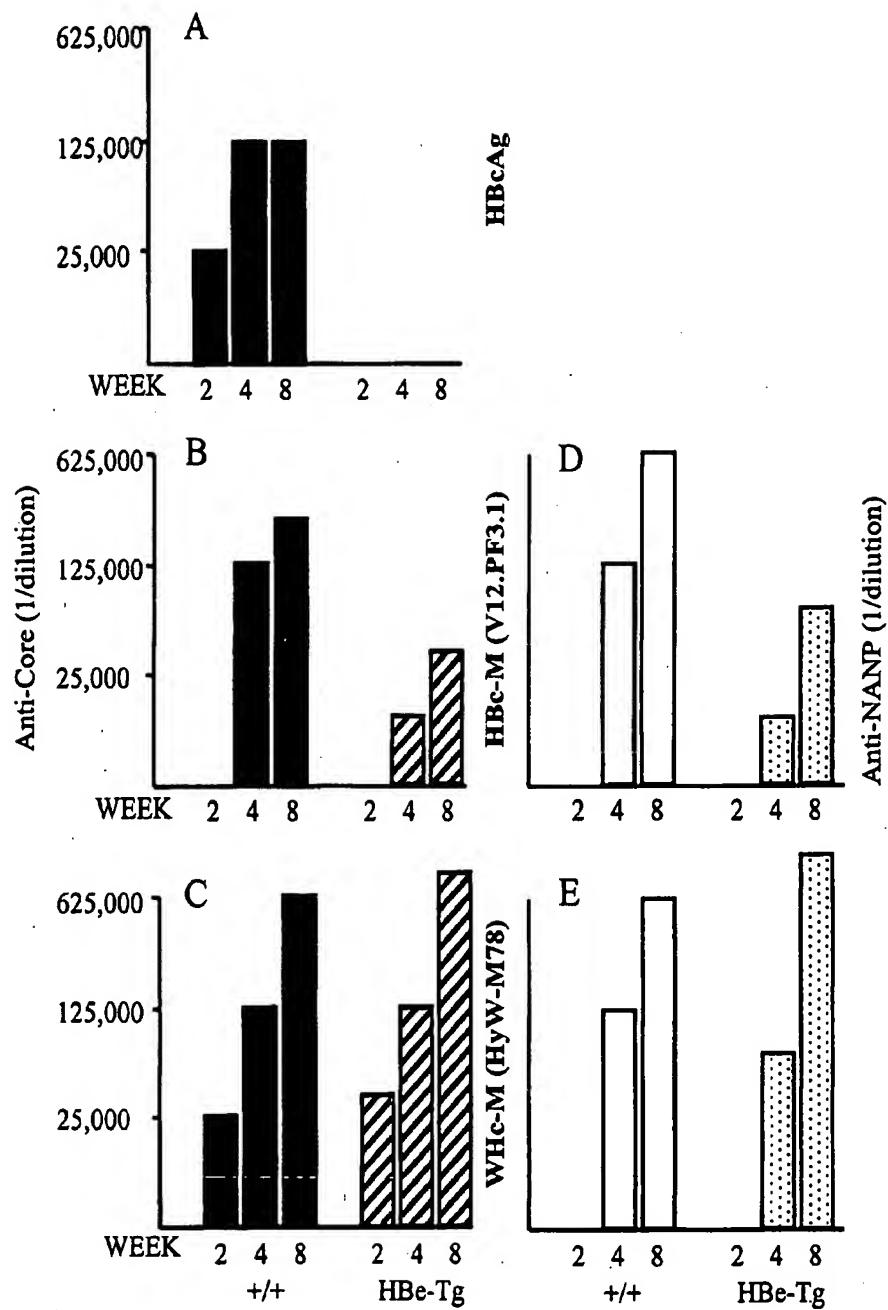


Fig. 12

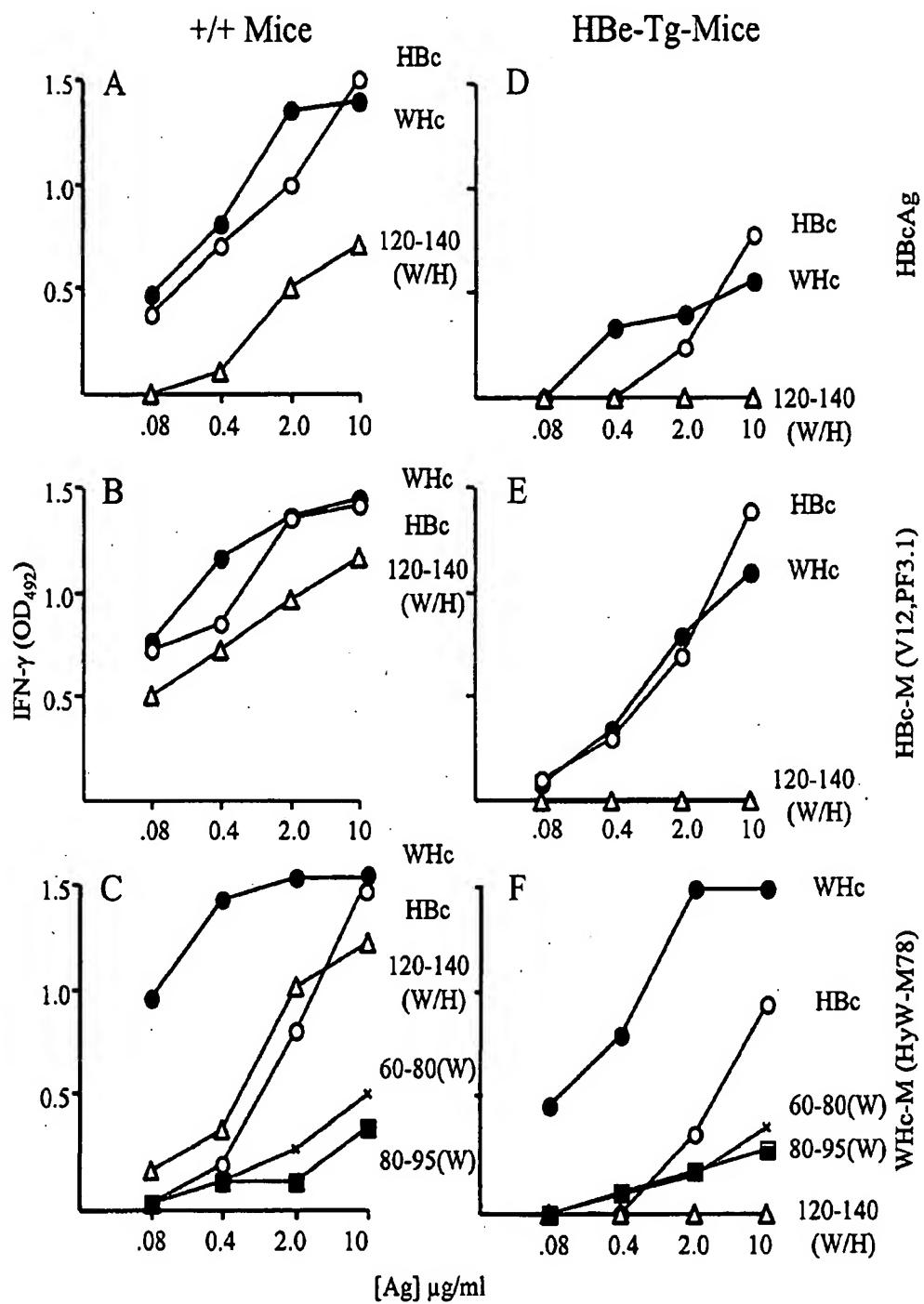


Fig. 13

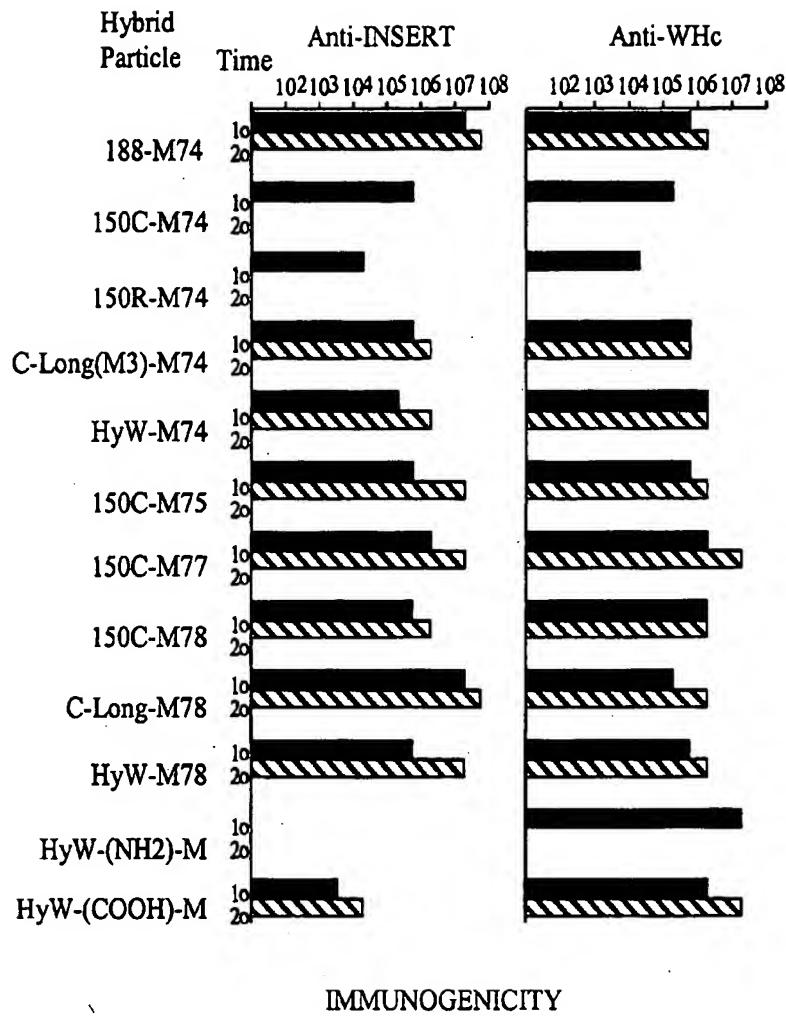


Fig. 14

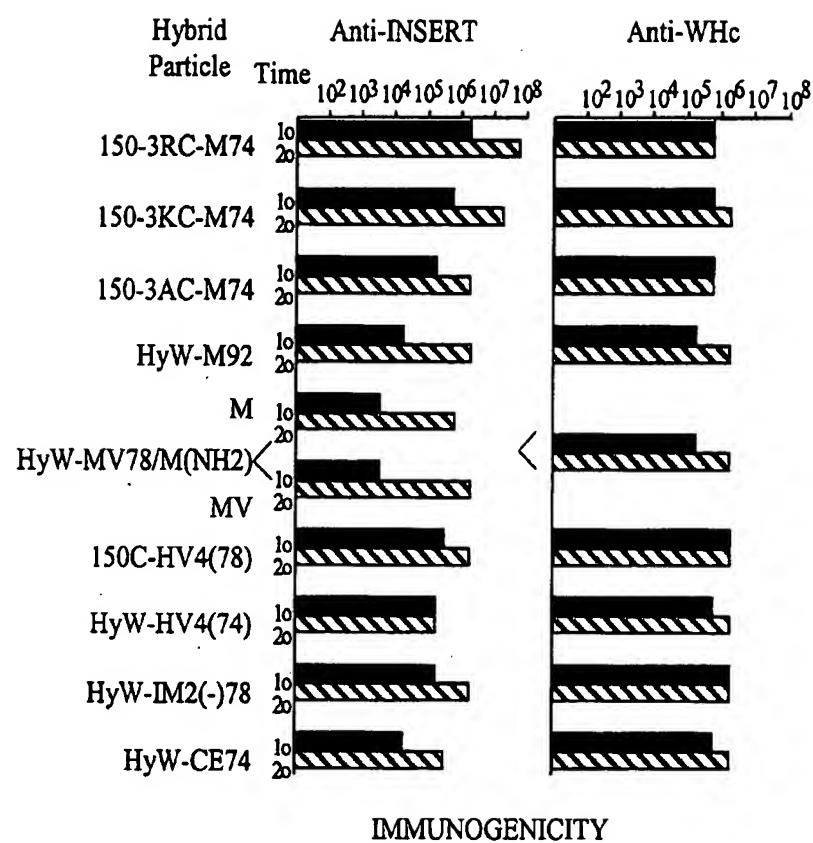


Fig. 15

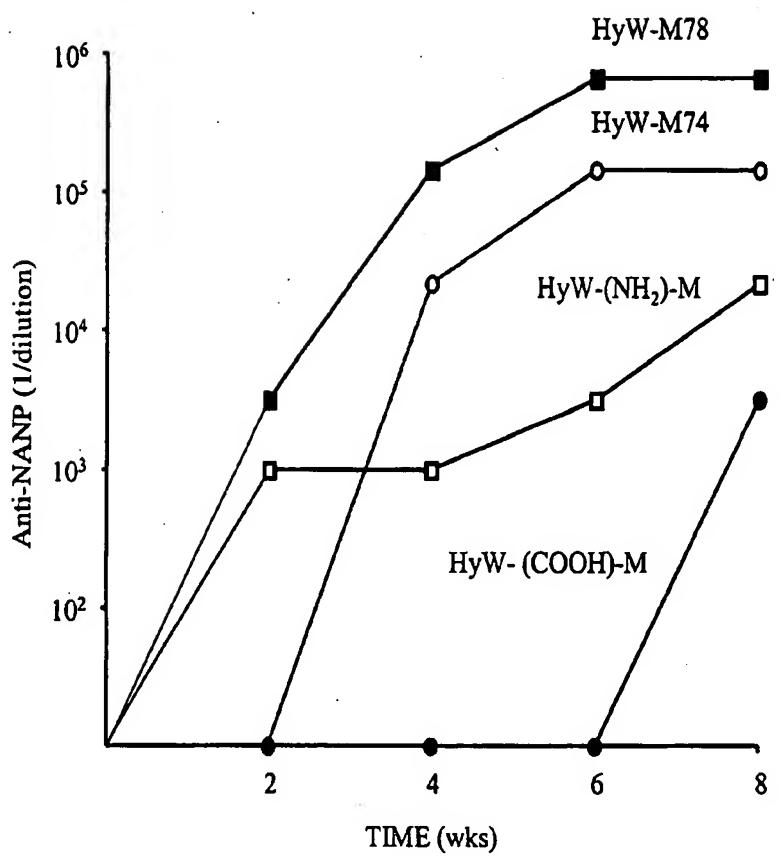


Fig. 16

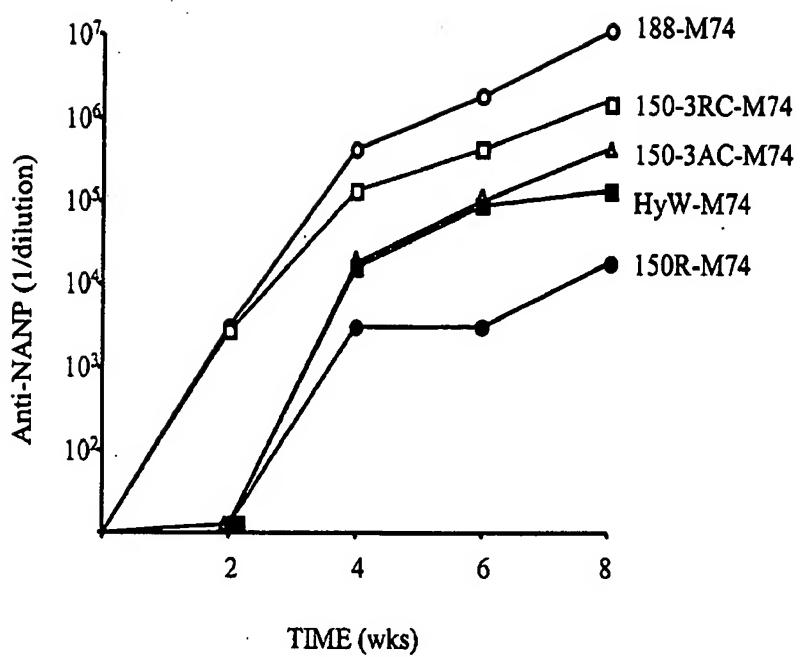
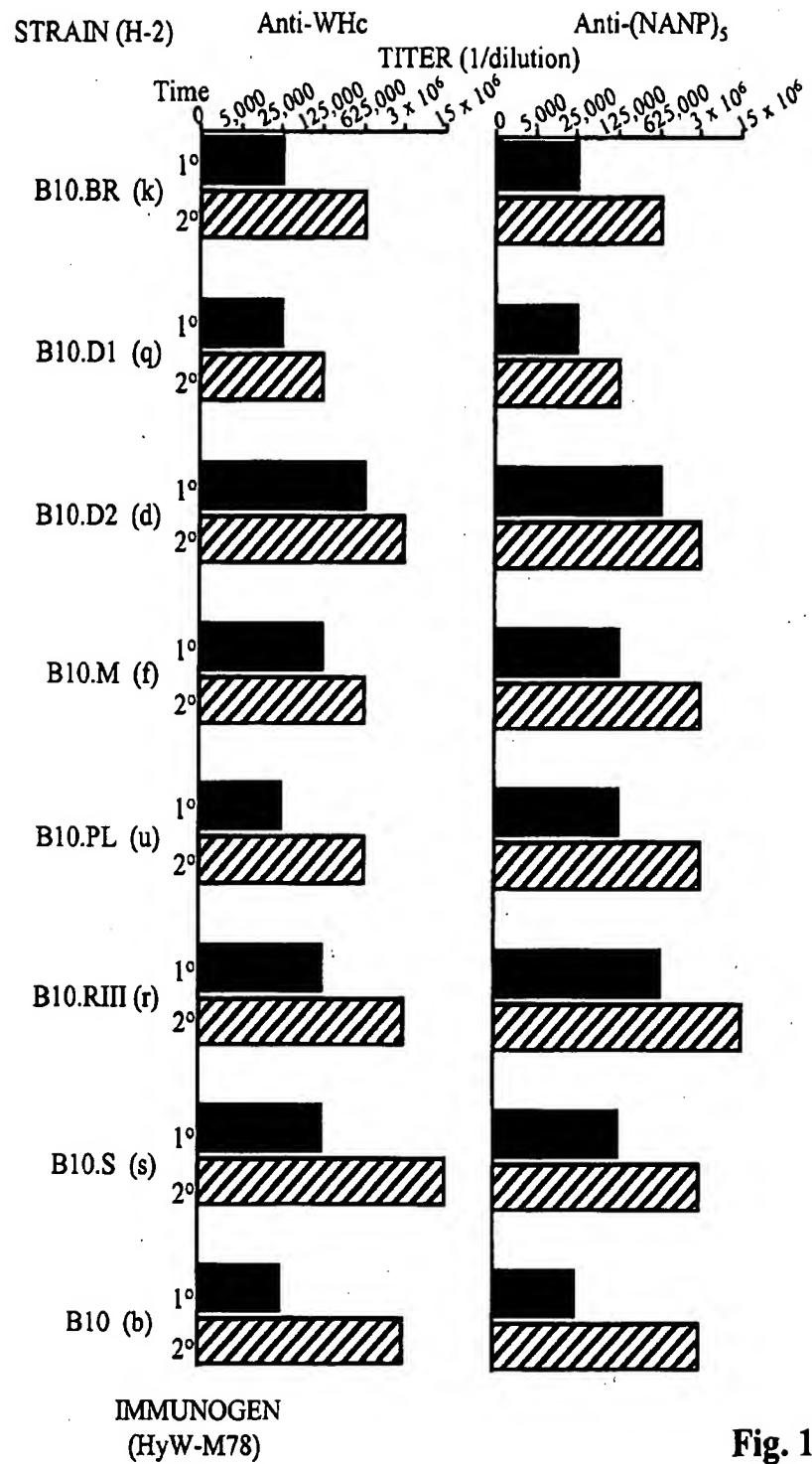


Fig. 17



**Fig. 18**

IMMUNOGEN

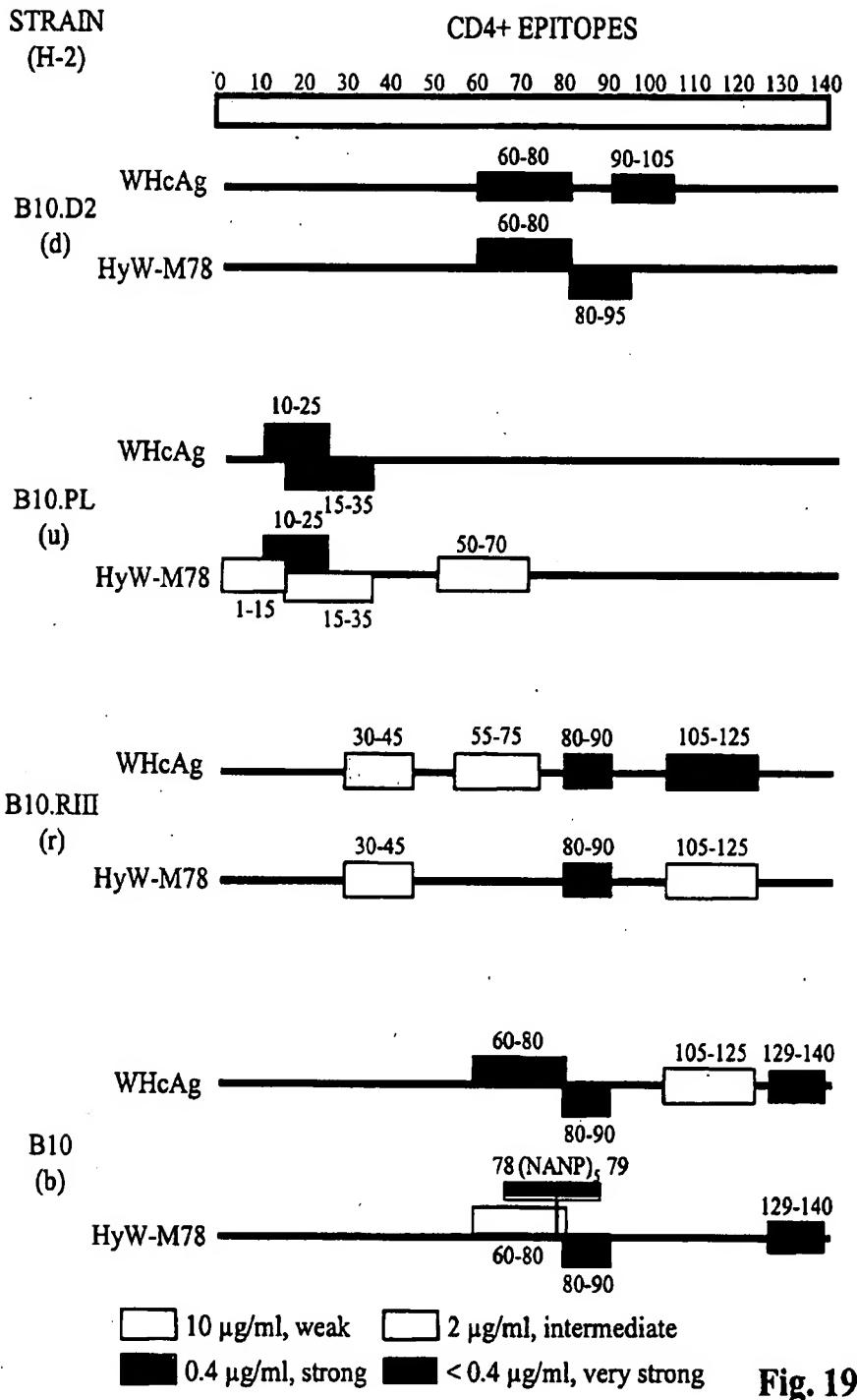
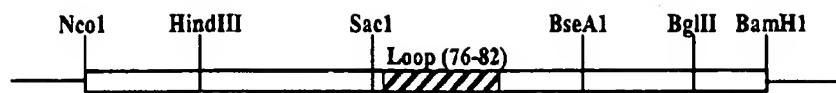
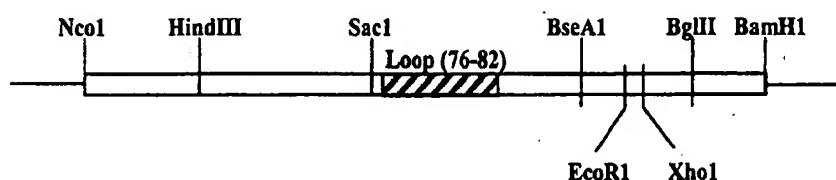
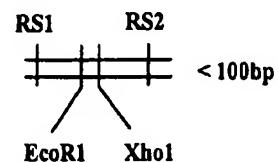


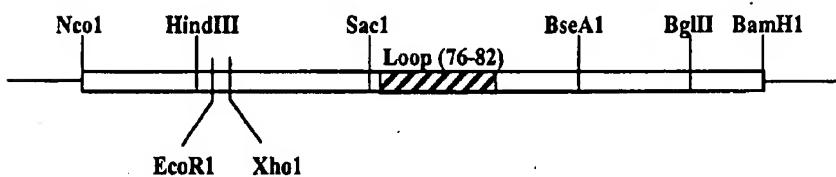
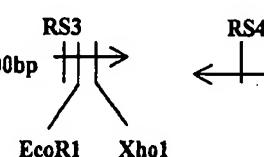
Fig. 19



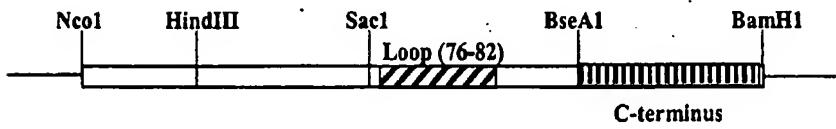
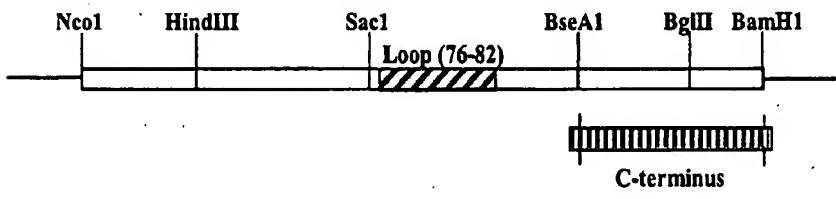
**A Direct Oligonucleotides Synthesis**



**B PCR**



**C C-Terminal Modification**



**Fig. 20**

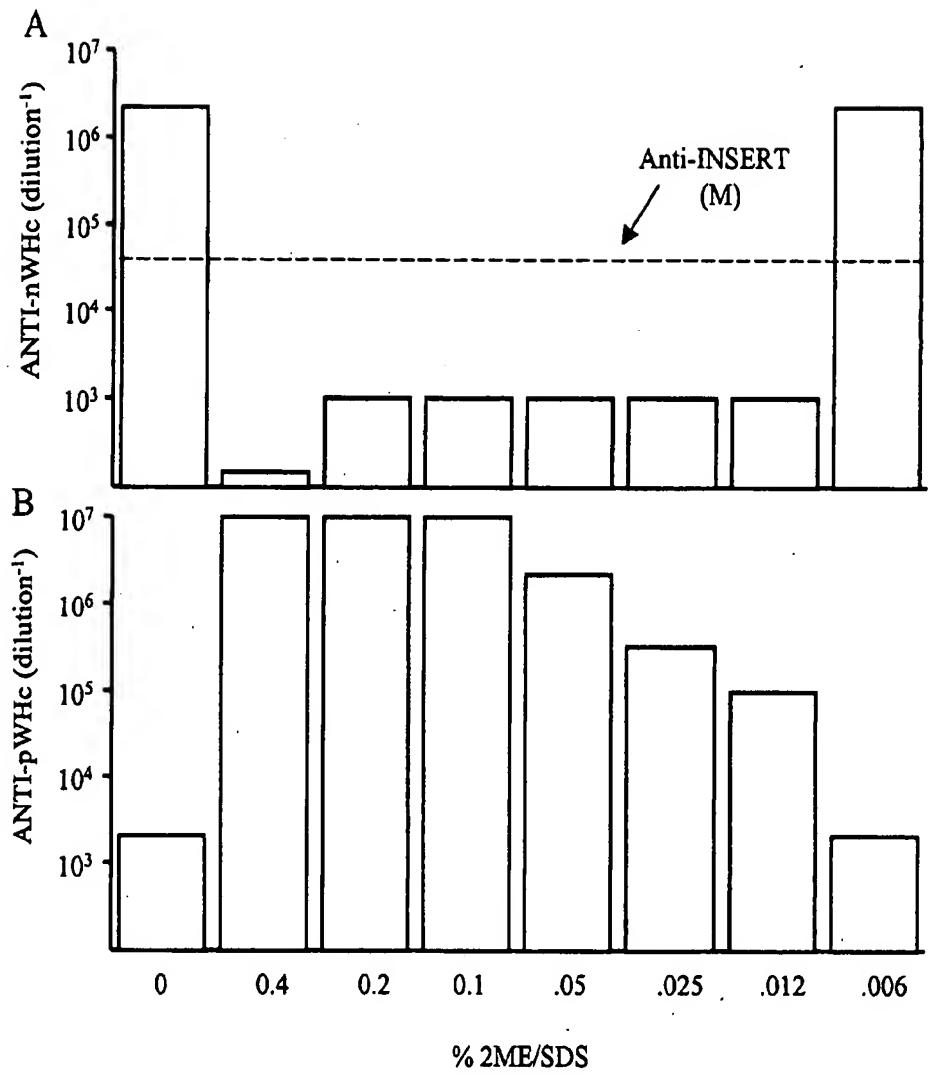
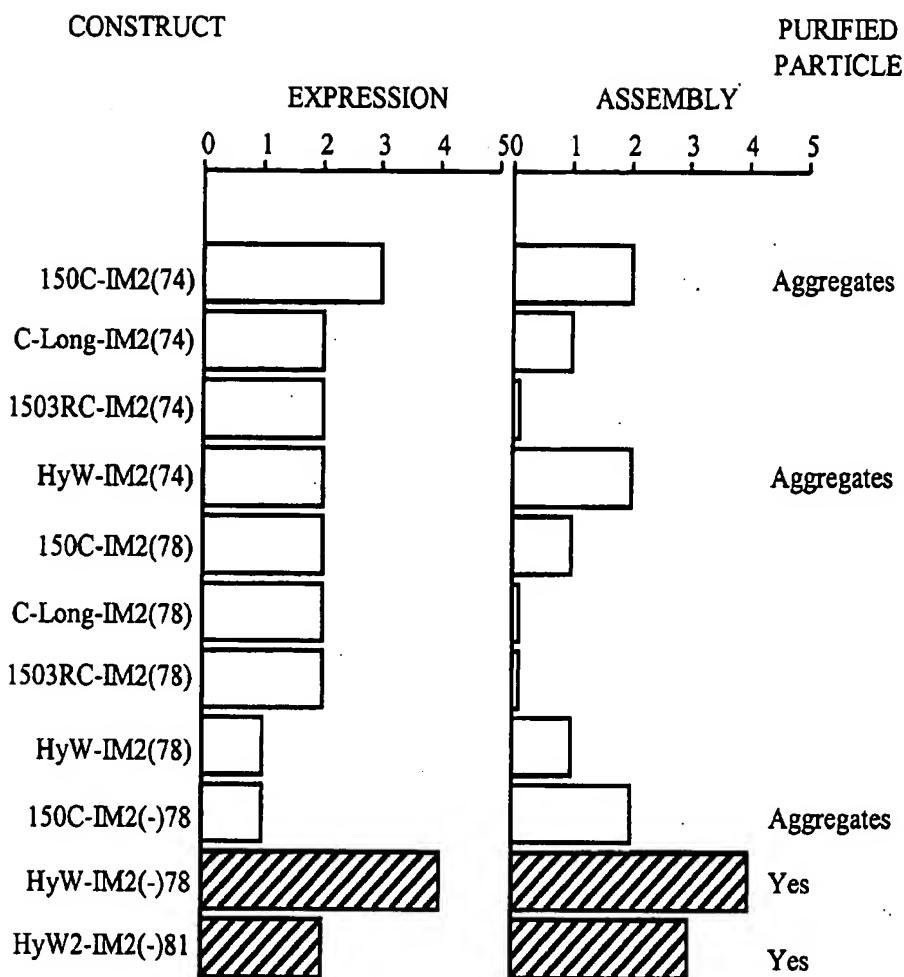


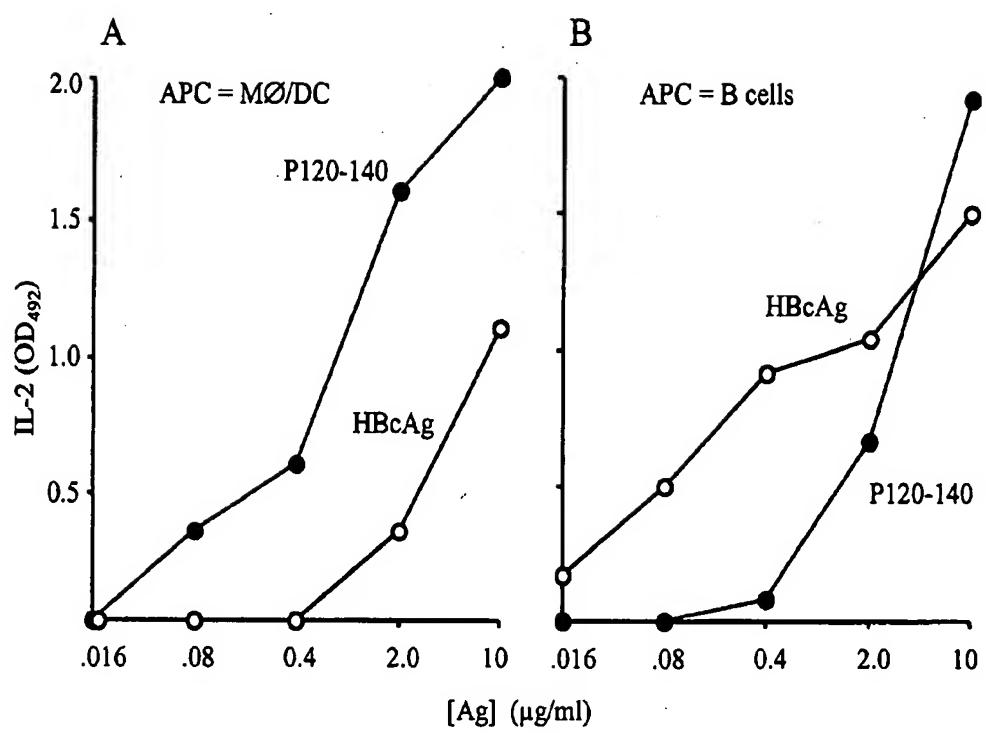
Fig. 21



**Fig. 22**

	Wt M2e	M S L L T E V E T P I R N E W G	C R C N D S S D	mAb 14C2	Polyclonal Anti-HyW-IM2(-)78
P1	- - - - -	- - - - -	- - - - -	51200	625000
P2	- - - - -	A - - - -	- - - - -	25600	125000
P3	- - - - -	- A - - -	- - - - -	12800	125000
P4	- - - - -	- - - - -	- - - - -	25600	$3 \times 10^6$
P5	- - - - -	- - - - -	- - - - -	6400	625000
P6	- - - - -	- - - - -	- - - - -	1600	625000
P7	- - - - -	- - - - -	A - - - -	12800	$3 \times 10^6$
P8	- - - - -	- - - - -	- A - - -	25600	625000
P9	- - - - -	- - - - -	- A - A -	102400	$3 \times 10^6$
Core-IM2(-) Particle	Core-M78 Particle	HyW-IM2(-)78		625000	15 x 10
			0		-
			(Dilution=0.5 OD <sub>492</sub> )	(1/Dilution)	

Fig. 23



**Fig. 24**

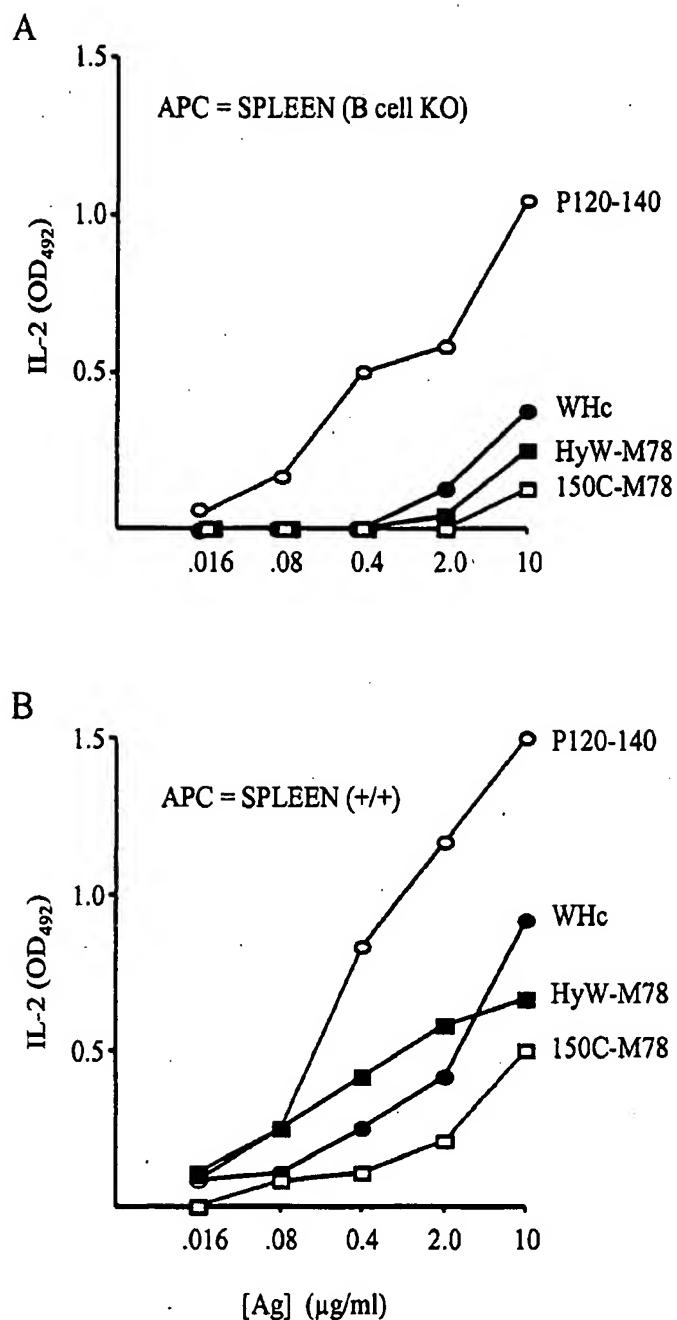


Fig. 25

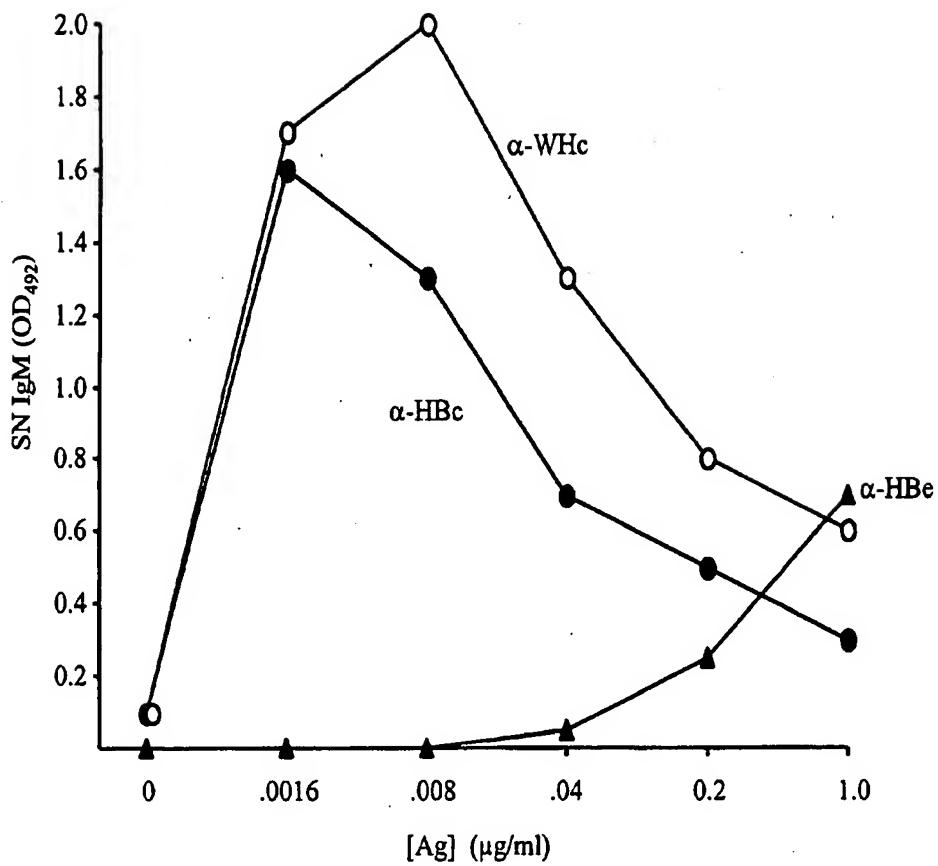


Fig. 26

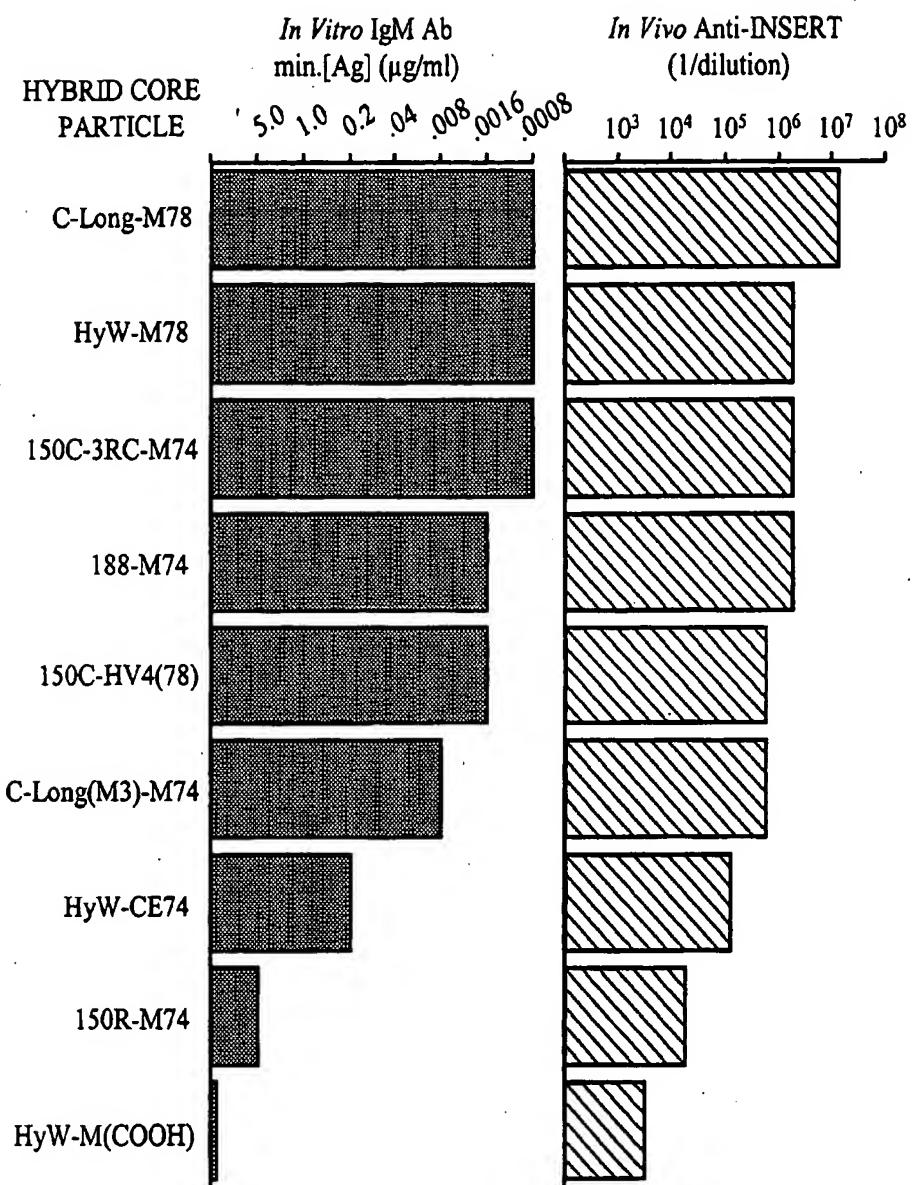


Fig. 27

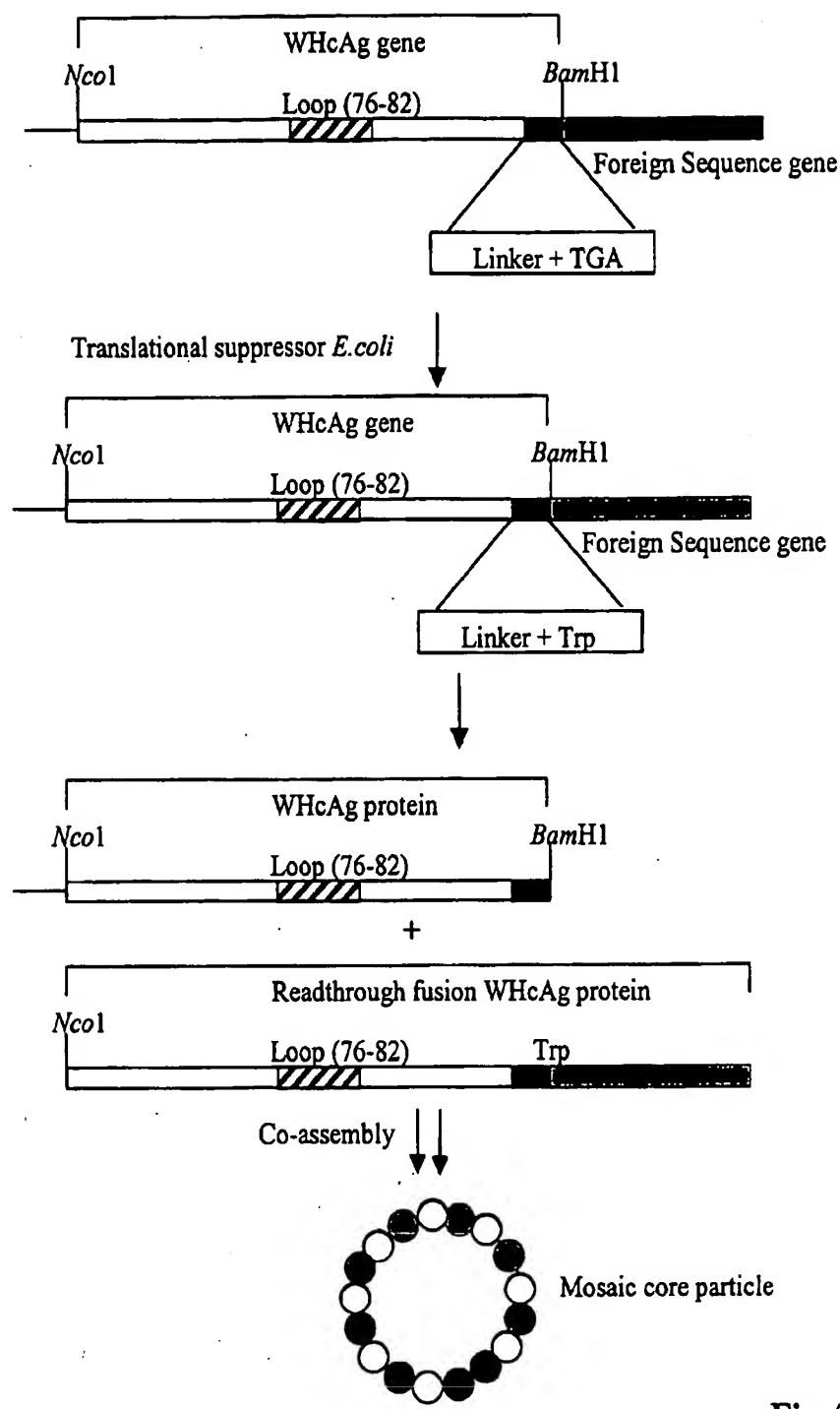


Fig. 28

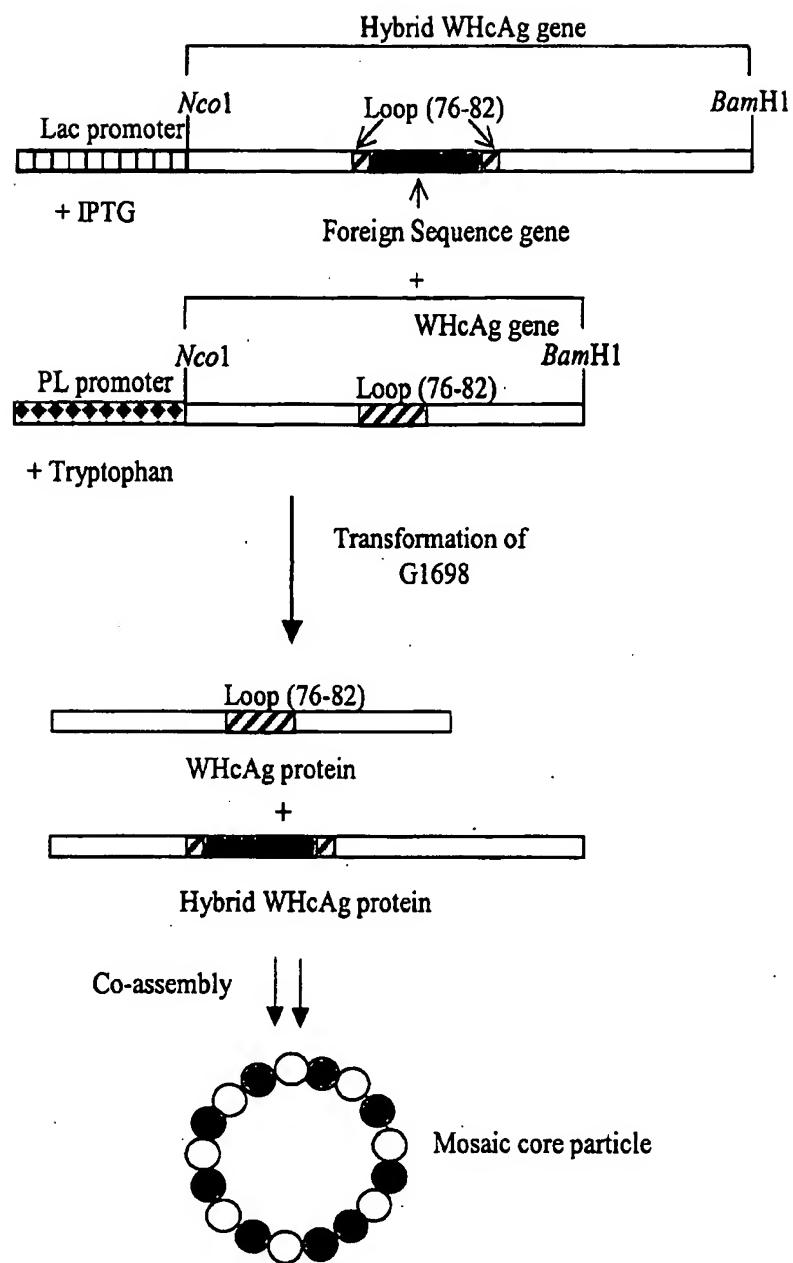
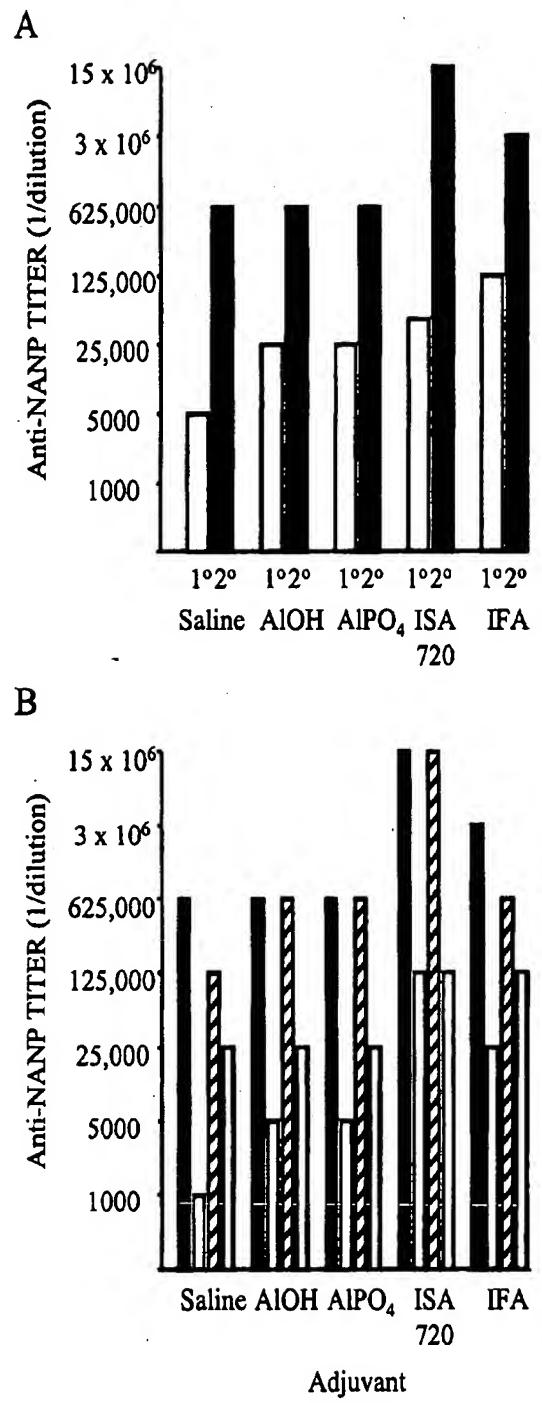
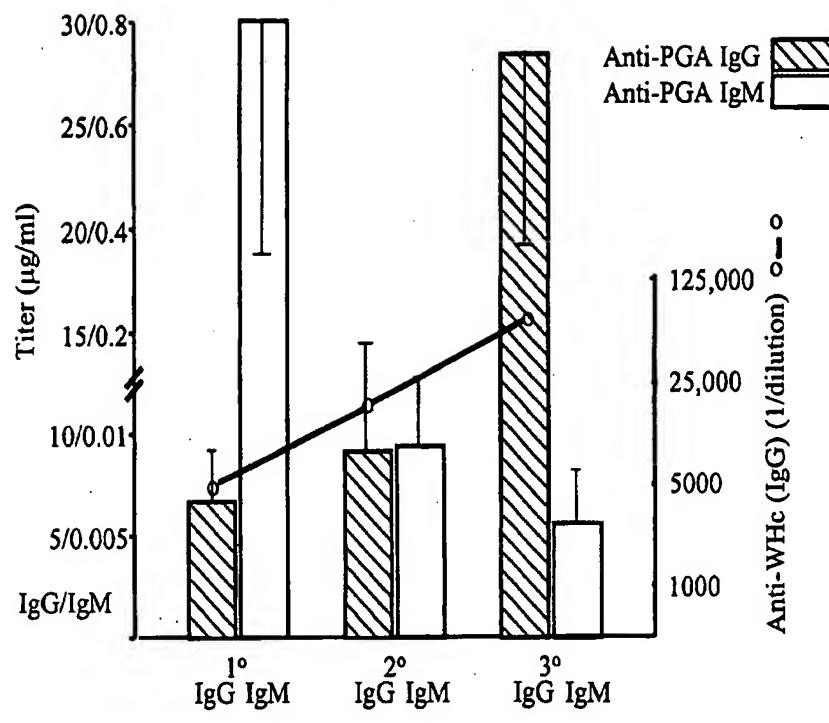


Fig. 29



**Fig. 30**



**Fig. 31**

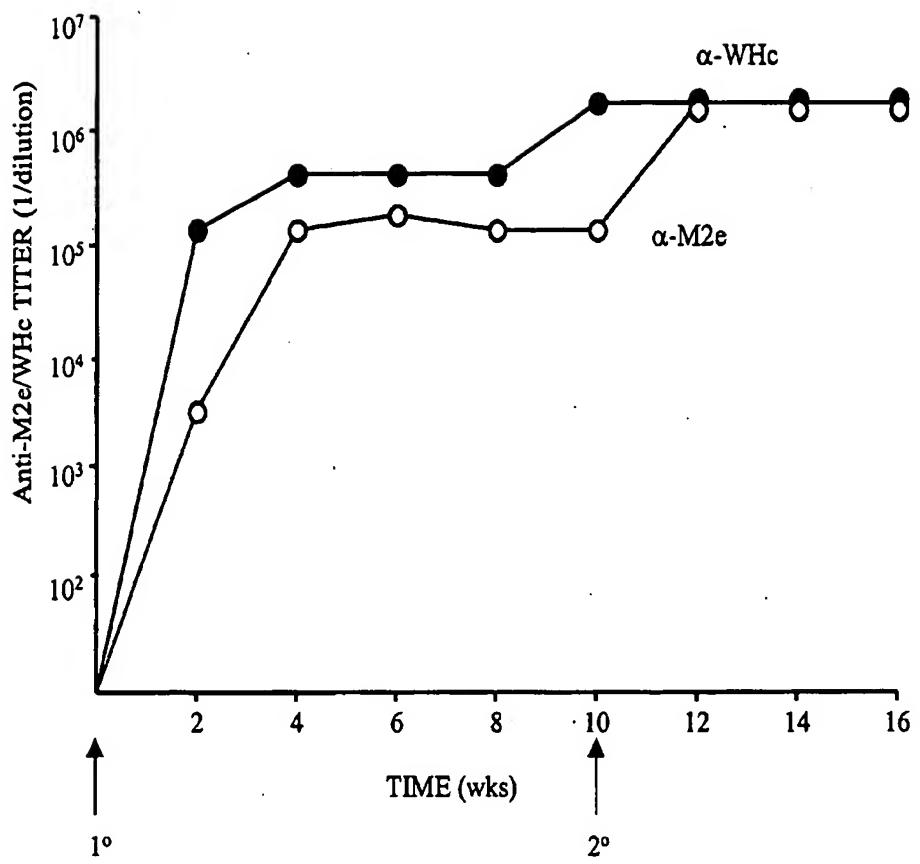


Fig. 32

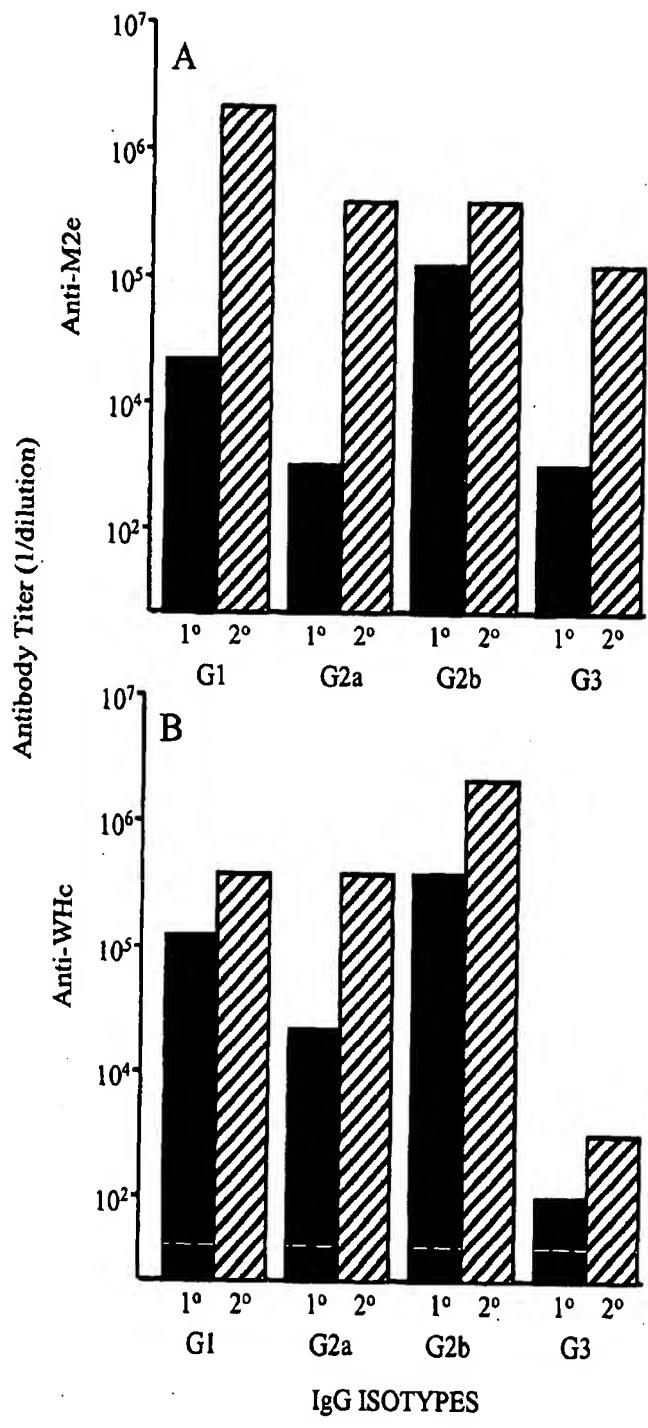


Fig. 33

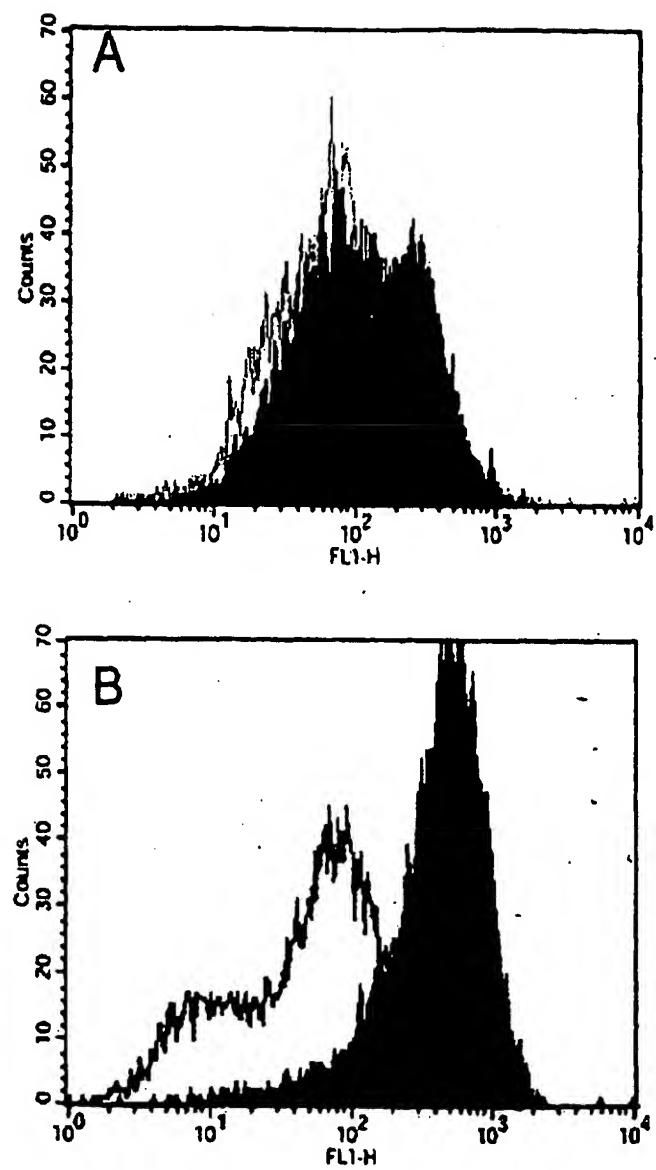
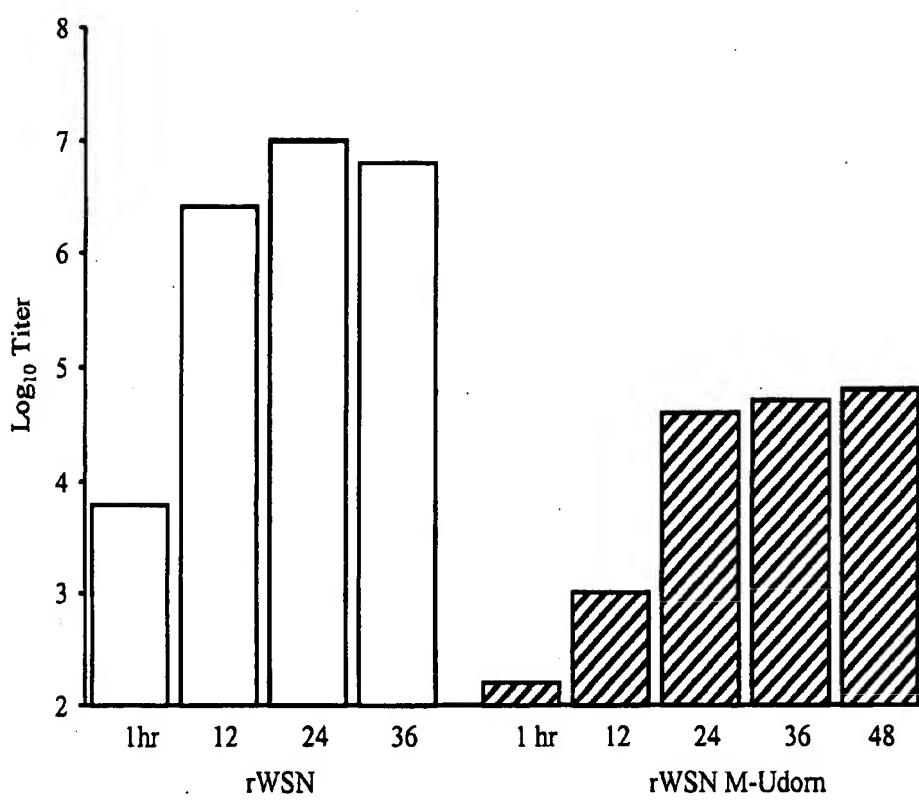


Fig. 34



**Fig. 35**

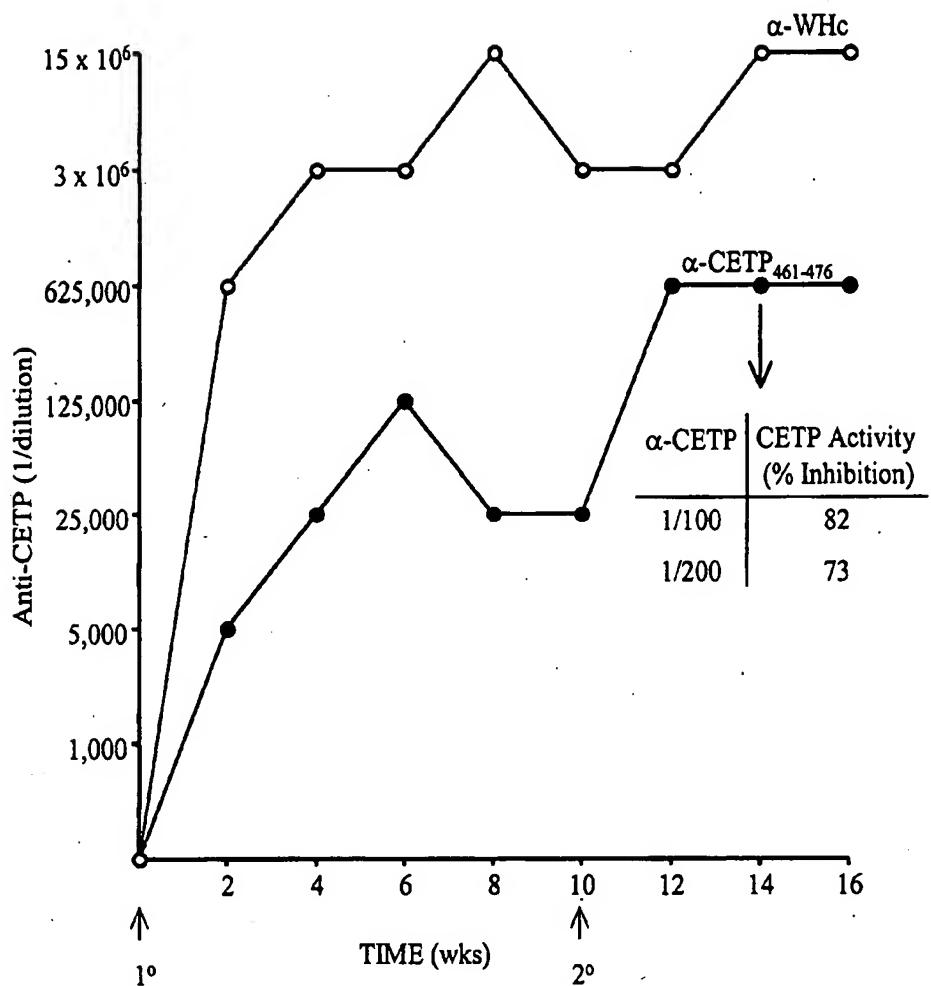
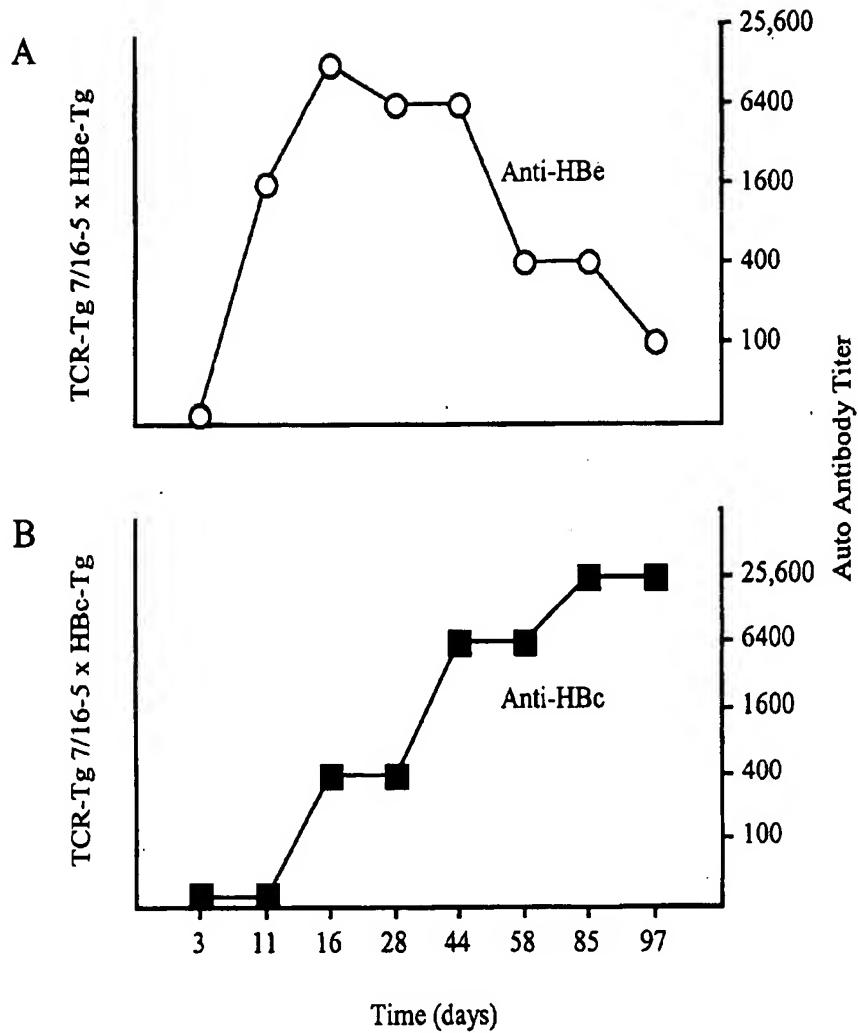


Fig. 36

**STRAIN**



**Fig. 37**

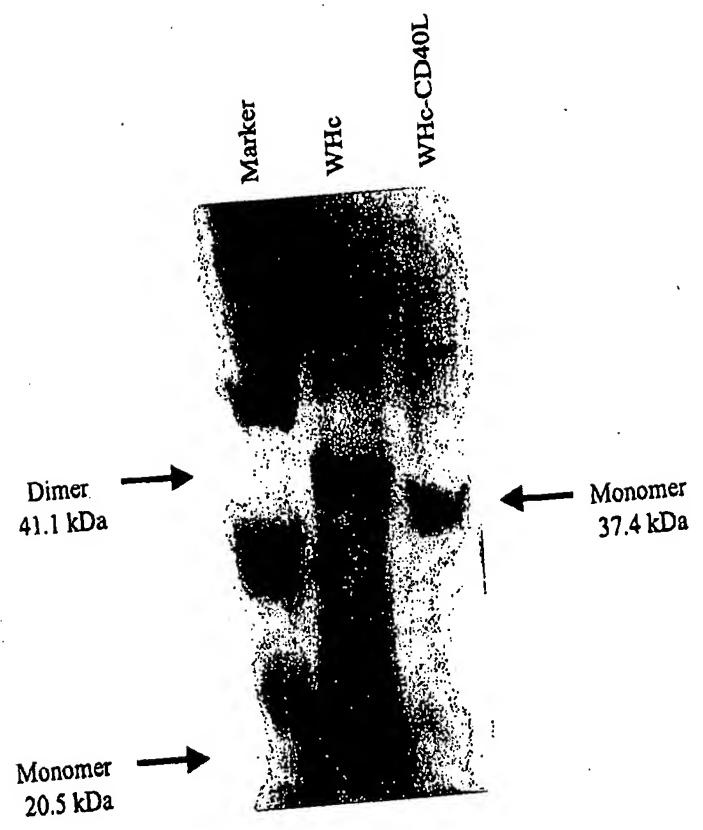
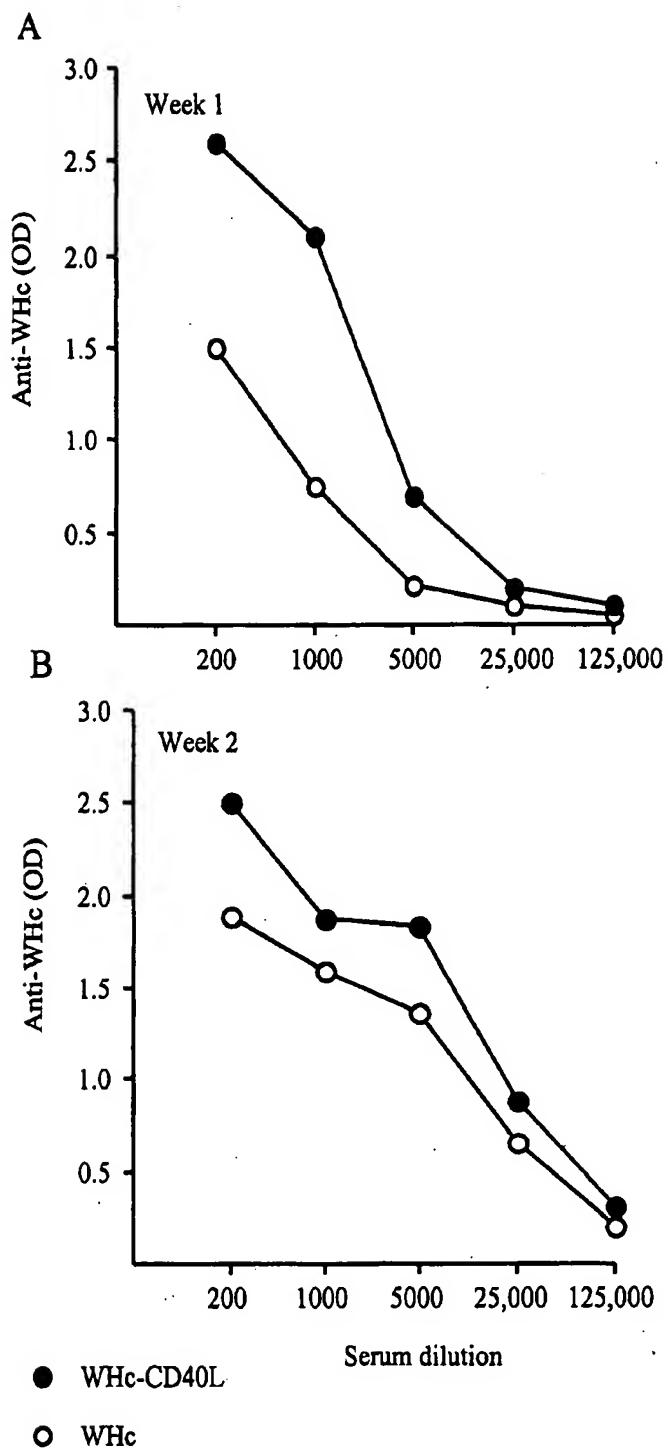


Fig. 38



**Fig. 39**

Fig. 40

A Wild Type WHcAg DNA (SEQ ID NO:37)

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TTGGACTTCTTCCTGACCTTAATGCTTGTTGGACACTGCTACTGCCCTGTATGAAG  
AAGAGCTAACAGGTAGGAACATTGCTCTCCGACCACAGCTATTAGACAAGCTTA  
GTATGCTGGGATGAATTAACAAATTGATAGCTTGGATGAGCTAACATAACTCTGA  
ACAAGTAAGAACAAATCATTGTAATCATGTCAATGATACCTGGGACTTAAGGTGAGAC  
AAAGTTTATGGTTCTTTGTCATTGTCATGTCCTCGGACAACATACAGTTCAAGAATT  
TTAGTAAAGTTGGAGTATGGATCAGGACTCCAGCTCCATATAGACCTCTAATGCACC  
CATTCTCTCGACTCTCCGGAACATACAGTCATTAGGAGAAGAGGAGGTGCAAGAGCTT  
CTAGGTCCCCAGAACAGACGACTCCCTCTCGCAGGAGAAGATCTCAATCACCGCGT  
CGCAGACGCTCTCAATCTCCATCTGCCAACTGCTGA

B Wild Type WHcAg (SEQ ID NO:1)

MDIDPYKEFGSSYQLLNFLPLDFFPDLNALVDTATALYEEELTGREHCSPHHTAIRQAL  
VCWDELTKLIAMMSSNITSEQVRTIIVNHVNDTWGLKVRQSLWFHLSCLTFQHTVQEF  
LVSFGVWIRTPAPYRPPNAPISTLPEHTVIRRRGGARASRSPRRRTPSPRRRSQSPR  
RRRSQSPSANC

C Truncated WHcAg (SEQ ID NO:38)

MDIDPYKEFGSSYQLLNFLPLDFFPDLNALVDTATALYEEELTGREHCSPHHTAIRQAL  
VCWDELTKLIAMMSSNITSEQVRTIIVNHVNDTWGLKVRQSLWFHLSCLTFQHTVQEF  
LVSFGVWIRTPAPYRPPNAPISTLPEHTVI

Fig. 41

A Wild Type GSHcAg DNA (SEQ ID NO:39)

```
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TTGGACTTTTCCTGATCTCAATGCATTGGTGGACACTGCTGCTCTTATGAAG
AAGAATTAACAGGTAGGGAGCATTGTTCTCCTCATCATACTGCTATTAGACAGGCC
GTGTGTTGGGAAGAATTAACTAGATTAATTACATGGATGAGTGAAAATACAACAGAAGA
AGTTAGAAGAATTATTGTTGATCATGTCAATAACTTGGGGACTTAAAGTAAGACAGA
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GTTAGTTTGGAGTATGGATTAGAACACTCCAGCTCCTTATAGACCACCTAATGCACCC
TTTATCAACTCTCCGGAACATACAGTCATTAGGAGAACAGGAGGTTCAAGAGCTGCTA
GGTCCCCCGAACAGCACACTCCCTCCTCGCAGGAGAACGGTCAATCACCGCGTCGC
AGACGCTCTCAATCTCCAGCTTCCAACGTGCTGA
```

B Wild Type GSHcAg (SEQ ID NO:21)

```
MDIDPYKEFGSSYQLLNFLPLDFFPDLNALVDTAAALYEEELTGREHCS
PHHTAIRQAL
VCWEELTRLITWMSENTTEEVRRRIIVDHVNNTWGLKVRQTLWFHLSCLTFQ
HTVQEFL
VSFGVWIRTPAPYRPPNAPILSTLPEHTVIRRRGGSRAARSPRRRTSP
RRRRSQSPRR
RRSQSPASNC
```

C Truncated GSHcAg (SEQ ID NO:40)

```
MDIDPYKEFGSSYQLLNFLPLDFFPDLNALVDTAAALYEEELTGREHCS
PHHTAIRQAL
VCWEELTRLITWMSENTTEEVRRRIIVDHVNNTWGLKVRQTLWFHLSCLTFQ
HTVQEFL
VSFGVWIRTPAPYRPPNAPILSTLPEHTVI
```

**Fig. 42**

**A Wild Type HBcAg DNA (SEQ ID NO:57)**

```
ATGGACATCGACCCTTATAAAAGAATTGGAGCTACTGTGGAGTTACTCTCGTTTGCC
TTCTGACTTCTTCCTCAGTACGAGATCTCTAGATACCGCCTCAGCTCTGTATCGGG
AAGCCTTAGAGTCTCCTGAGCATTGTCACCTCACCACTGCACTCAGGCAAGCAATT
CTTGCTGGGGGAACTAATGACTCTAGCTACCTGGGTGGTAAATTGGAAGATCC
AGCATCCAGAGACCTAGTAGTCAGTTATGTCAACACTAATATGGGCCTAAAGTCAGGC
AACTCTTGTGGTTCACATTCTGTCTCACTTTGGAAGAGAAACCGTTATAGAGTAT
TTGGTGTCTTCGGAGTGTGGATTGCACTCCTCCAGCTTATAGACCAACCAAATGCC
TATCCTATCAACACTCCGGAAACTACTGTTAGACGACGAGGCAAGGTCCCCTAGAA
GAAGAACTCCCTGCCTCGCAGACGAAGGTCTCAATGCCCGTCGAGAAGATCTCAA
TCTCGGAATCTCAATGTTGA
```

**B Wild Type HBcAg (SEQ ID NO:41)**

```
MDIDPYKEFGATVELLSFLPSDFFPSVRDLDDTASALYREALESPEHCSPHHTALRQAI
LCWGELMTLATWGVNLEDPASRDLVVSYVNNTMGLKFRQLLWFHISCLTFGRETVIEY
LVSFGWIRTPPAYRPPNAPILSTLPETTVVRRGRSPRRRTSPRRRSQSPRRRSQ
SRESQC
```

**C Truncated HBcAg (SEQ ID NO:58)**

```
MDIDPYKEFGATVELLSFLPSDFFPSVRDLDDTASALYREALESPEHCSPHHTALRQAI
LCWGELMTLATWGVNLEDPASRDLVVSYVNNTMGLKFRQLLWFHISCLTFGRETVIEY
LVSFGWIRTPPAYRPPNAPILSTLPETTVV
```

## DECLARATION FOR PATENT APPLICATION

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated next to my name. I believe I am an original, first and joint inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled **Human Hepatitis B Virus Core Proteins As Vaccine Platforms And Methods Of Use Thereof**, the specification of which is attached hereto. I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, § 1.56(a).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under § 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full Name of Sole or First Inventor: **David R. Milich**

Inventor's Signature: _____	Date: _____
Residence: <u>15434 Sky High Road, Escondido, California, 92025</u>	Citizenship: <u>United States</u>
Post Office Address: <u>15434 Sky High Road, Escondido, California, 92025</u>	

Full Name of Second Joint Inventor: **Jean-Noel Billaud**

Inventor's Signature: _____	Date: _____
Residence: <u>13056 Signature Point #137, San Diego, California, 92130</u>	Citizenship: <u>France</u>
Post Office Address: <u>13056 Signature Point #137, San Diego, California, 92130</u>	

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Application of: David R. Milich *et al.* Group No.:  
Serial No.:  
Filed:  
Entitled: Human Hepatitis B Virus Core Proteins As Vaccine Platforms And Methods  
Of Use Thereof

**POWER OF ATTORNEY BY ASSIGNEE**

Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Vaccine Research Institute of San Diego, as Assignee of record of the entire interest of the above-identified patent application, hereby appoints the members of the firm of MEDLEN & CARROLL, LLP, a firm composed of:

Jaen Andrews	(Reg. No. 35,051)	David A. Casimir	(Reg. No. 42,395)
Tanya A. Arenson	(Reg. No. 47,391)	Maha A. Hamdan	(Reg. No. 43,655)
Jason R. Bond	(Reg. No. 45,439)	Thomas C. Howerton	(Reg. No. 48,650)
Thomas J. Bordner	(Reg. No. 47,436)	J. Mitchell Jones	(Reg. No. 44,174)
Mary Ann D. Brow	(Reg. No. 42,363)	Christine A. Lekutis	(Reg. No. 51,934)
Thomas W. Brown	(Reg. No. 50,002)	David J. Wilson	(Reg. No. 45,225)
Peter G. Carroll	(Reg. No. 32,837)		

as its attorneys with full power of substitution to prosecute this application and transact all business in the Patent and Trademark Office in connection therewith.

Please direct all future correspondence and telephone calls regarding this application to:

Maha A. Hamdan  
MEDLEN & CARROLL, LLP  
101 Howard Street, Suite 350                      Telephone: 415/904-6500  
San Francisco, California 94105                      Facsimile: 415/904-6510

I hereby certify that the Assignment document filed with the application or filed subsequent to the filing date of the application, has been reviewed and I hereby certify that, to the best of my knowledge and belief, title is with Vaccine Research Institute of San Diego.

Dated: \_\_\_\_\_ By: \_\_\_\_\_

Name: \_\_\_\_\_

Title: \_\_\_\_\_

Vaccine Research Institute of San Diego  
3030 Science Park Road, Suite 100  
San Diego, California, 92121

**PATENT**

## **ASSIGNMENT**

WHEREAS, WE, David R. Milich and Jean-Noel Billaud, hereinafter referred to as "ASSIGNOR", have invented certain new and useful improvements as described and set forth in the below-identified application for United States Letters Patent:

**Title of Invention:** **Human Hepatitis B Virus Core Proteins As Vaccine Platforms And Methods Of Use Thereof**

**Filing Date:** **Serial No.:**

WHEREAS, Vaccine Research Institute of San Diego, a California corporation, 3030 Science Park Road, Suite 100, San Diego, California, 92121, hereinafter referred to as "ASSIGNEE", is desirous of acquiring the entire right, title and interest in said invention and application and in any Letters Patent which may be granted on the same;

NOW THEREFORE, TO ALL WHOM IT MAY CONCERN: Be it known that, for and in consideration of the sum of One Dollar (\$1.00) lawful money paid to ASSIGNOR by ASSIGNEE and other good and valuable consideration, receipt of which is hereby acknowledged, ASSIGNOR has sold, assigned and transferred, and by these presents does sell, assign and transfer unto said ASSIGNEE, and ASSIGNEE's successors and assigns, all right, title and interest in and to said invention, said application for United States Letters Patent, and any Letters Patent which may hereafter be granted on the same in the United States and all countries throughout the world including any divisions, renewals, continuations in whole or in part, substitutions, conversions, reissues, prolongations or extensions thereof, said interest to be held and enjoyed by said ASSIGNEE as fully and exclusively as it would have been held and enjoyed by said ASSIGNOR had this assignment and transfer not been made, to the full end and term of any Letters Patent.

ASSIGNOR further agrees that ASSIGNOR will, without charge to said ASSIGNEE, but at ASSIGNEE's expense, cooperate with ASSIGNEE in the prosecution of said application and/or applications; execute, verify, acknowledge and deliver all such further papers, including applications for Letters Patent and for the reissue thereof, and instruments of assignment and transfer thereof; and will perform such other acts as ASSIGNEE lawfully may request, to obtain or maintain Letters Patent for said invention and improvement in any and all countries, and to vest title thereto in said ASSIGNEE, or ASSIGNEE's successors and assigns.

IN TESTIMONY WHEREOF, ASSIGNOR has hereunto signed ASSIGNOR's names to this assignment on the date indicated below.

David R. Milich

Jean-Noel Billaud

STATE OF \_\_\_\_\_ )  
COUNTY OF \_\_\_\_\_ )  
SS.

On this \_\_\_\_\_ day of \_\_\_\_\_, in the year of \_\_\_\_\_, before me, the undersigned notary public, personally appeared the above-named ASSIGNOR, known to me (or proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument, and acknowledged that he/she executed the same.

**NOTARY PUBLIC**

**SEAL**

## **ABSTRACT**

The present invention relates to hepatitis virus core proteins and nucleic acids. In particular, the present invention provides compositions and methods comprising recombinant hepatitis virus core proteins or nucleic acids for use in vaccine formulations.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

MS Patent Application  
Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

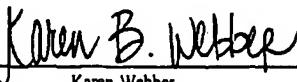
NEW APPLICATION TRANSMITTAL

Transmitted herewith for filing is the patent application of David R. Milich and Jean-Noel Billaud for Human Hepatitis B Virus Core Proteins As Vaccine Platforms And Methods Of Use Thereof.

CERTIFICATION UNDER 37 C.F.R. § 1.10

I hereby certify that this New Application Transmittal and the documents referred to as enclosed therein are being deposited with the U.S. Postal Service on this date July 30, 2003 in an envelope as "Express Mail Post Office to Addressee" Mailing Label Number EV 329 476 681 US addressed to: MS Patent Application, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

10/630074 PTO  
03917 U.S. 07/30/03



Karen Webber

1. Type Of Application

This new application is for a(n)

Original (nonprovisional)

2. Papers Enclosed That Are Required For Filing Date Under 37 C.F.R. § 1.53(b) (Regular) or 37 C.F.R. § 1.153  
(Design) Application

111 Pages of Specification

4 Pages of Claims

1 Page of Abstract

42 Sheets of Formal Drawings

3. Declaration

Enclosed

Unexecuted.

4. Inventorship Statement

The inventorship for all the claims in this application is:

the same

5. Language

English

6. Assignment

An assignment of the invention to Vaccine Research Institute of San Diego is attached.

Form PTO-159S will follow.

7. Fee Calculation (37 C.F.R. § 1.16)

Regular application

CLAIMS AS FILED

Number Filed	Number Extra	Rate	Basic Fee - \$750.00 (37 C.F.R. § 1.16(a))
Total Claims (37 C.F.R. § 1.16(c))	30 - 20 =	10 × \$18.00 =	\$180.00
Independent Claims (37 C.F.R. § 1.16(b))	4 - 3 =	1 × \$84.00 =	\$84.00
Multiple Dependent Claim(s), if any (37 C.F.R. § 1.16(d))		+ \$280.00 =	\$0.00
Filing Fee Calculation			\$1014.00

8. Small Entity Statement(s)

Verified Statement(s) that this is a filing by a small entity under 37 C.F.R. §§ 1.9 and 1.27.

Filing Fee Calculation (50% of above) \$507.00

PATENT  
Attorney Docket No.: VACCINE-07971

9. Fee Payment Being Made At This Time

Enclosed

basic filing fee

\$507.00

Total Fees Enclosed

\$507.00

10. Method of Payment of Fees

Check in the amount of \$507.00

11. Authorization To Charge Additional Fees and Credit Overpayment

The Commissioner is hereby authorized to charge payment of any fees associated with this communication or credit any overpayment to Deposit Account No.: 08-1290. An originally executed duplicate of this transmittal is enclosed for this purpose.

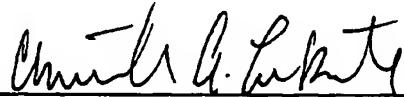
12. Power of Attorney by Assignee

Enclosed

13. Return Receipt Postcard

Enclosed

Dated: July 30, 2003



Christine A. Lekutis  
Registration No.: 51,934

Please direct future inquiries to:

Maha A. Hamdan  
Registration No.: 43,655

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Statement Where No Further Pages Added  
 This transmittal ends with this page.